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ACTION OF STREPTOCOCCAL HAEMOLYSINS AND PROTEOLYTIC ENZYMES ON EHRLICH ASCITES TUMOUR CELLS*

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It has been recently shown that various strains of *Streptococcus pyogenes* group A possess a cell-bound haemolysin (CBH) which can be released from the streptococcal cells into the surrounding medium by some surface active materials (Tween 40, Tween 80, Triton) by crystalline albumin, but not by sonic energy (Ginsburg and Grossowicz, 1957; Ginsburg, 1958; Ginsburg and Grossowicz, 1959). This haemolytic factor was designated as streptolysin "D" (SLD) D signifying detergent.

SLD is distinguished from streptolysin O (SLO) streptolysin S (SLS) (Todd, 1938) and from the intracellular haemolysin (IH) described by Schwab (Schwab, 1956) on the bases of different substrate requirements for formation, sensitivity to U.V. irradiation and to sonic energy (Ginsburg, 1958; Ginsburg and Grossowicz, unpublished).

Besides haemolysing RBC of various animal species both cell-free and cell-bound SLD, are also capable of injuring and killing various mammalian cells *in vitro* (Ehrlich ascites tumour cells, fibroblasts, amnion cells, leucocytes) (Ginsburg, 1958; Ginsburg and Grossowicz, 1959).

The purpose of the present study is to show that Ehrlich ascites tumour cells damaged by different streptococcal haemolysins may be disintegrated by various proteolytic enzymes which by themselves are not lethal.

MATERIALS AND METHODS

Streptococcal strain.—A Lancefield group A *Strep. pyogenes* (strain S84 type 3) was obtained from the State Serum Institute, Copenhagen. The streptococcus was cultivated in Brain Heart Infusion (BHI) broth (Difco).

Preparation of washed streptococci.—Streptococci grown in BHI medium for various periods of time at 37° were washed 3 times with 20 ml. (final volume 60 ml.) of buffered saline

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(equal volumes of phosphate buffer 0·067 M, pH-7·4 and saline). The washed streptococci were diluted to contain approximately 5×10^8 cells/ml. and were put in ice until used.

Haemolysins

Cell-bound haemolysin (*CBH*) was obtained by incubating washed streptococci (5×10^8 cells/ml. obtained from a 6 hr culture) for 5 min. in the presence of glucose, $MgSO_4$, $7H_2O$ and cysteine free-base (0·5 mg./ml. of each). Under such conditions a potent haemolytic activity appeared in close association with the streptococcal cells,ho wever no extra-cellular haemolysin was obtained. When however 25 mg./ml. of salt-free crystalline blood albumin (Cutter laboratories) were added to the streptococci the CBH was released into the surrounding medium. This haemolysin was designated as streptolysin D (SLD). CBH and SLD were assayed according to the method of Slade and Knox (1950). Haemolytic activity is expressed as haemolytic units/ml.—HU/ml.

Streptolysin O (*SLO*).—Supernatants obtained from streptococcal cultures grown for 18 hr. in BHI were used as a source of haemolysin. These supernatants were incubated for 10 min. at 37° with 1 mg./ml. of cysteine in order to secure maximal activation of SLO. SLO was titrated according to the method of Slade and Knox (1950).

Streptolysin S (*SLS*).—Was prepared and assayed according to the method of Bernheimer (1949).

Proteolytic enzymes

Crystalline streptococcal proteinase—was kindly supplied by Dr. S. D. Elliot, Department of Animal Pathology, School of Veterinary Medicine, Madingley Road, Cambridge, England.

The enzyme was activated with 0·1 per cent cysteine before conducting the experiments. The enzyme solution always contained insoluble material which was removed by centrifugation.

Crystalline trypsin—(Tryptar) was purchased from the Armour Laboratories, Illinois.

Papain (Nutrition Biochemical Corporation Cleveland Ohio).—The enzyme was activated with 0·1 per cent cysteine before the experiment was conducted. Insoluble material was removed by centrifugation.

All enzymes were dissolved in buffered saline and were assayed by the method of Clifton and Cannamela (1953).

EXPLANATION OF PLATES

All photographs were taken with a Spencer Phase contrast microscope.

FIG. 1.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate-saline buffer containing glucose (500 μg./ml.) $MgSO_4 . 7H_2O$ (500 μg./ml.) and cysteine (500 μg./ml.). × 500.

FIG. 2.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate saline buffer containing glucose, Mg^{++} and cysteine and 5×10^8 cells/ml. of streptococci (strain S84) possessing 1000 H.U./ml. of SLD. At the left lower corner of the picture streptococcal chains are seen attached to tumour cells. × 500.

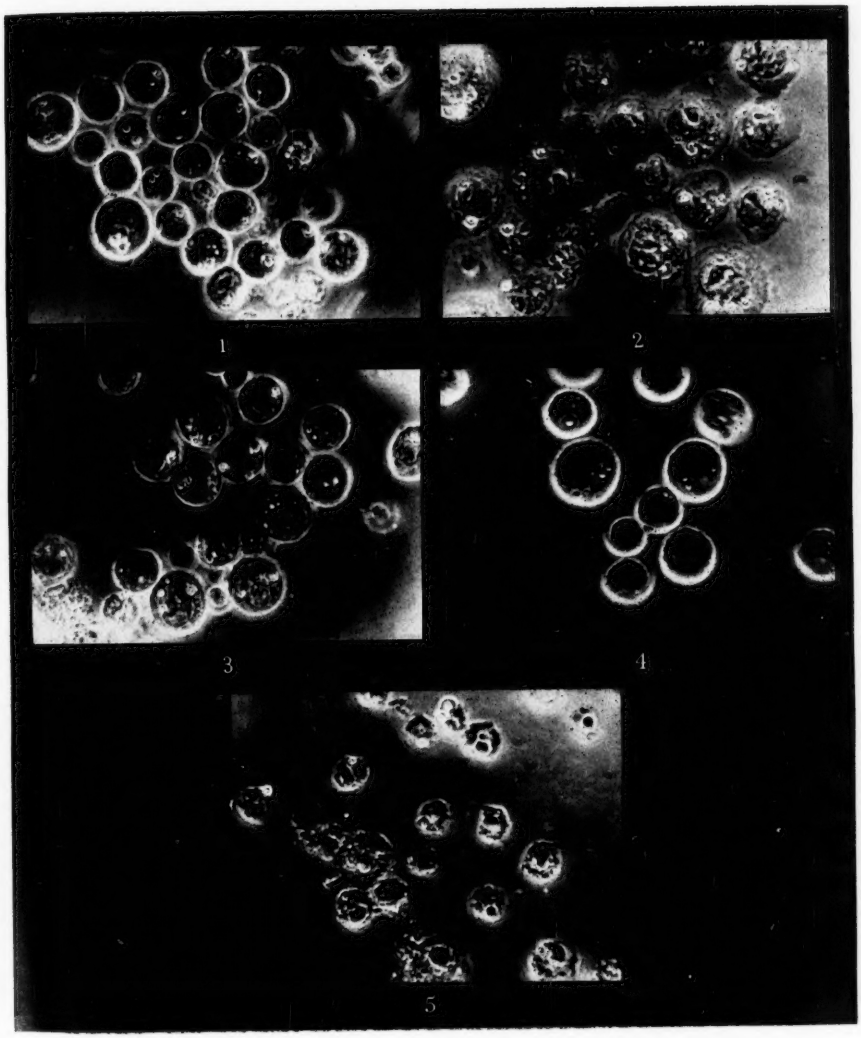
FIG. 3.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate saline buffer containing glucose Mg^{++} cysteine, 5×10^8 cells/ml. of streptococci and 25 μg./ml. of aureomycin. The streptococci were preincubated for 10 min. at 37° with aureomycin before the mixture was added to tumour cells. Aureomycin was found to abolish the haemolytic activity of the streptococci. × 500.

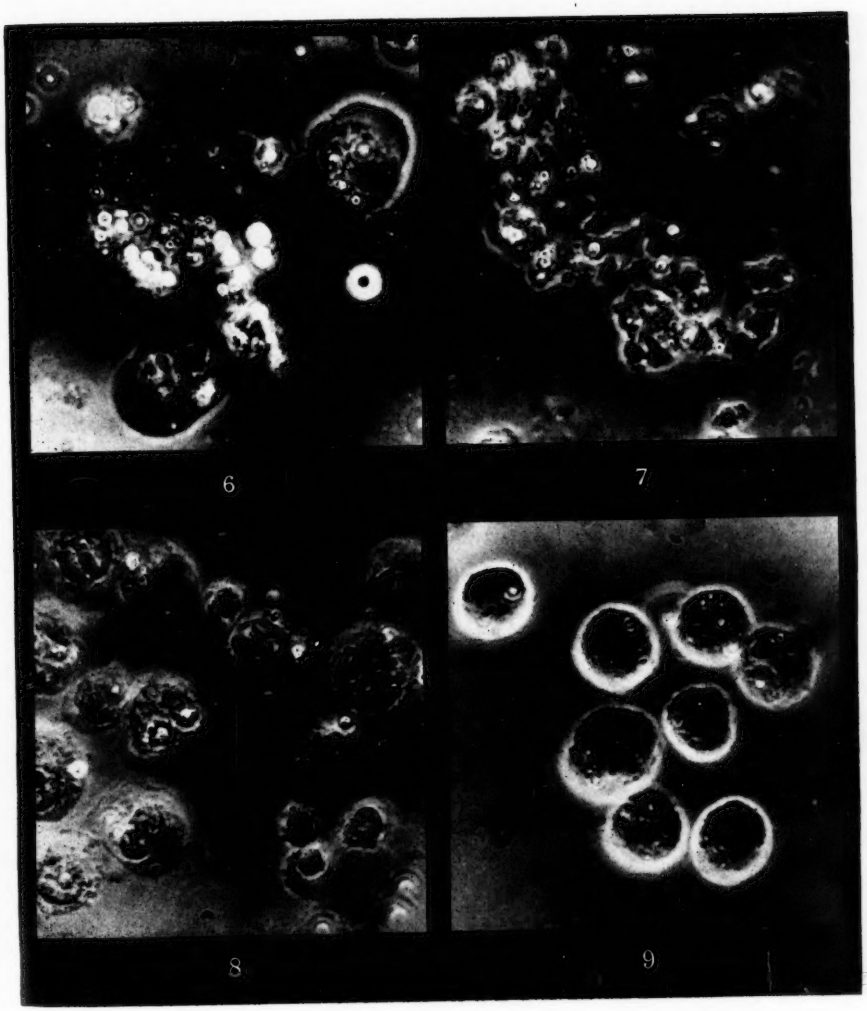
FIG. 4.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate saline buffer containing glucose, Mg^{++}, cysteine and 100 μg./ml. of streptococcal proteinase. × 500.

FIGS. 5, 6, 7.—Ehrlich tumour cells incubated for 30, 60 and 90 minutes respectively, at 37° in the presence of phosphate saline buffer containing glucose Mg^{++}, cysteine, streptococci (5×10^8 cells/ml.—possessing 1000 H.U./ml. SLD), and 100 μg./ml. of streptococcal proteinase. *Note* the various stages of cell disintegration and the accumulation of cell debris. (Fig. 5. × 500. Figs. 6 and 7 × 710).

FIG. 8.—Ehrlich tumour cells incubated for 90 min. at 37° with SLD (1000 H.U/ml.). Similar results were obtained with SLO, and SLS. × 710.

FIG. 9.—Ehrlich tumour cells incubated for 90 min. at 37° with SLD (1000 H.U./ml.), trypan blue 25 μg./ml. and proteinase (100 μg./ml.). Trypan blue was found to abolish the haemolytic activity of cell-free SLD. (Ginsburg, 1958). × 710.





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Ehrlich ascites tumour cells

Ehrlich tumour cells were obtained from mice infected with the Landchutz strain. The tumour cells were maintained by weekly passages in white mice. The ascites fluid was removed from mice killed by decapitation. The cells were washed with buffered saline and diluted to contain approximately 10^7 cells/ml.

RESULTS

Action of streptococci possessing CBH and streptococcal proteinase on Ehrlich ascites tumour cells

All experiments on the action of streptococcal haemolysins and proteolytic enzymes on tumour cells were performed at 37° and were recorded by direct observations with a phase microscope.

Untreated tumour cells.—Untreated cells (7 days old) (Fig. 1) appeared round, the edge of the cells smooth, the nucleus when visible was surrounded by a narrow rim of cytoplasm and only few granules could be seen scattered in the cytoplasm. Such cell suspensions always contained 2–3 per cent of dead cells as demonstrated by vital staining with trypan blue (2 per cent). A minimum of 500 cells were counted in each measurement.

Tumour cells incubated with streptococci.—The tumour cells (10^7/ml) began to swell 10 min. after the addition of streptococci (5×10^8/ml.) possessing CBH activity (1000 H.U./ml.). The nucleus became more obvious and many glistening cytoplasmic granules appeared in the swollen cells. The average diameter of most of the cells was twice that of controls 90 min. after the addition of streptococci (Fig. 2). The nucleus was swollen and some streptococcal chains were attached to the swollen tumour cells. Only 20 per cent viable cells were present as shown by staining with trypan blue. No cell disintegration or cell debris could be demonstrated and no reduction in the total number of cells could be observed even after 90 min. of incubation (Table I).

The swelling and the death of the cells could not be prevented by ascitic fluid, rabbit sera, or by penicillin (1000 u/ml.) but could be prevented to a large extent by aureomycin (25 μg./ml.). Aureomycin was found to inhibit CBH production (Ginsburg and Grossowicz, 1957). Tumour cells treated with streptococci-aureomycin mixtures appeared somewhat swollen and contained no more than 5 per cent of dead cells.

Tumour cells incubated with streptococcal proteinase.—The concentrations of proteinase used in these experimens (10–100 μg./ml., 0·2–2 u/ml.) had no visible effect on the tumour cells for the first 60 min. of the experiment. After further incubation (90 min.) some cells appeared somewhat swollen (Fig. 4). However, no change in the total number of the cells could be demonstrated (Table I). Staining with trypan blue revealed that only 2–4 per cent of dead cells were present.

The combined action of streptococci and of proteinase on tumour cells.—The first signs of cell disintegration could be observed 30 min. after the addition of streptococcal proteinase to streptococci-tumour mixtures (Fig. 5). Most of the swollen cells still contained eccentric nuclei which appeared distorted. The cytoplasm which appeared empty and swollen was surrounded by a cell membrane, and many free nuclei were seen floating in the medium. After 60 min. of incubation many free globules appeared in the medium together with large accumula-

TABLE.—*The Combined Action of Haemolytic Streptoccci Possessing CBH and Proteinase on Ehrlich Ascites Tumour Cells*

Reaction mixture Tumour cells† +	Number of cells: Before treatment	Number of cells: After treatment	Percentage of cells disappearing*
	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
streptococci‡	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
streptococci+aureomycin§ (25 μg./ml.)	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
proteinase (25 μg./ml.)	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
proteinase (50 μg./ml.)	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
proteinase (100 μg./ml.)	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
proteinase (100 μg./ml.)+ascitic fluid‖	$1{\cdot}4\times10^7$	$2{\cdot}5\times10^6$	82
streptococci+proteinase (25 μg./ml.)	$1{\cdot}4\times10^7$	$1{\cdot}7\times10^6$	88
streptococci+proteinase (50 μg./ml.)	$1{\cdot}4\times10^7$	$6{\cdot}8\times10^5$	95
streptococci+proteinase (100 μg./ml.)	$1{\cdot}4\times10^7$	$7{\cdot}3\times10^6$	48
streptococci+proteinase (25 μg./ml.)+ascitic fluid	$1{\cdot}4\times10^7$	$5{\cdot}4\times10^6$	62
streptococci+proteinase (50 μg./ml.)+ascitic fluid	$1{\cdot}4\times10^7$	$4{\cdot}4\times10^6$	68
streptococci+proteinase (100 μg./ml.)+ascitic fluid	$1{\cdot}4\times10^7$	$1{\cdot}4\times10^7$	—
streptococci+proteinase (100 μg./ml.)+aureomycin (25 μg./ml.)+ascitic fluid			

* The figures are the average of 3 experiments. All cells that still possessed a cell membrane, no matter how damaged they appeared, were counted. Cell counts were performed after 90 minutes of incubation at 37°.

† $1{\cdot}4\times10^7$ cells/ml. suspended in phosphate saline buffer.

‡ Strain S84 (type 3)—5×10§ cells/ml. obtained from a 6 hr culture grown in BHI medium. The cells were washed with phosphate saline buffer and were resuspended in buffered saline containing glucose $MgSO_4 . 7H_2O$ and cysteine (0·5 mg./ml.) of each and incubated for 5 min. at 37° before they were added to tumour cells. The cells possessed 1000 H.U./ml. of CBH.

§ Aureomycin was found to abolish the haemolytic activity of the streptococci. Streptococci were incubated for 5 min. at 37° with aureomycin before they were added to tumour cells.

‖ Supernatant obtained from Ehrlich tumour cells. 0·5 ml. of undiluted fluid were added to 0·5 ml. of reaction mixture.

tions of cell debris (Fig. 6). A few swollen cells showing pseudopod-like structures could still be seen. The process of disintegration advanced rapidly, and 90 min. after the addition of streptococcal proteinase (100 μg./ml.) over 95 per cent of the cells disappeared (Table I). The rest of the cells were in various stages of disintegration (Fig. 7). When however the production of CBH was blocked by aureomycin, no cell disintegration or reduction in the total number of cells occurred upon addition of proteinase, and only few cells appeared somewhat swollen (Table I).

Changes in cells were obtained even in the presence of 50 per cent ascitic fluid or rabbit's serum, however the percent of cells remaining was somewhat higher than that obtained in the absence of body fluid and was roughly proportional to the amount of proteinase present (Table I).

Dilute streptococcal suspensions ($< 10^4$ cells/ml.) which after 60 min. of incubation had no effect on the cells, in the presence of proteinase caused marked swelling and some disintegration. These experiments suggested that CBH and proteinase act synergistically.

The action of SLD, SLO, SLS and proteinase on Ehrlich tumour cells.—The above experiments were repeated using SLD, SLO, SLS (1000 H.U./ml., 500 H.U./ml. and 1000 H.U./ml. respectively). Similar results were obtained when tested in washed system. The cells that were in contact with the various haemolysins swelled (Fig. 8) and upon addition of proteinase (100 μg./ml.) marked

disintegration of the cells occurred. This effect was completely inhibited by anti-SLD (Fig. 9), SLO and SLS agents (trypan blue, in the case of SLD and SLS (Ginsburg, 1958; Bernheimer, 1954) cholesterol and rabbit serum (Van Heyningen, 1950) and ascitic fluid in the case of SLO).

The effect of SLD, SLS and various other proteolytic enzymes on Ehrlich tumour cells.—Crystalline trypsin (10 μg./ml.–1 u/ml.) and crude papain (50 μg./ml.–1 u/ml.) were capable of disintegrating tumour cells previously injured by streptococcal haemolysins (SLD, SLS) when tested in washed systems. On the addition of ascitic fluid or rabbit serum the cells did not disintegrate but did show considerable swelling. The possibility that this effect was due to the inhibition of the proteolytic enzymes by the body fluids (Shulman, 1952) was then tested. It was found that 1 ml. of ascitic fluid or rabbit serum could inhibit the fibrinolytic activity of 1000 μg. of trypsin, 500 μg. of papain but only about 20 μg. of streptococcal proteinase. These results explain why low concentrations of trypsin, papain capable of acting on washed injured cells are inactive in the presence of body fluids. If however the concentrations of trypsin and papain were sufficiently high (1500 μg./ml.) cell disintegration occurred.

The mechanism of the cytotoxic-pathological effects observed was further investigated by exposing tumour cells to hypotonic solutions (0·5–0·6 per cent NaCl). This treatment caused swelling of the cells similar to that obtained by haemolysin treatment, on the further addition of small amounts of trypsin or papain (50 μg./ml.) marked disintegration of the cells occurred. (Quantitation of the remaining cells is hampered by the fact that trypsin and papain cause the agglutination of the tumour cells into a sticky mass which traps both intact cells and cell debris.) These concentrations of proteolytic enzymes had no effect on cells in isotonic medium. This would suggest that the primary damaging action of the haemolysin is on the cell membrane.

DISCUSSION

The results show that living Ehrlich tumour cells injured by various streptococcal haemolysins can be disintegrated by various proteolytic enzymes of animal, plant or streptococcal origin. The mechanisms of the action of these 2 types of agents either singly or in combination is little known. The fact that the haemolytic agents induced changes in cells expressed by marked swelling of the cells, by pseudopod-like formation and by the death of the cells suggests that as in the case of red blood cells the permeability and integrity of the cell membrane has been impaired.

As to action of proteolytic enzymes on living cells, it is interesting to note that relatively high concentrations of these enzymes (*e.g.* trypsin, papain) are routinely used for tissue culture purposes without causing any apparent permanent damage to the cells, since they can be easily propagated further (Chang, 1954; Younger, 1954).

The data also indicate that there is a synergistic effect of streptococcal proteinase and various streptococcal haemolysins (SLO, SLS, SLD) on tumour as well as on normal cells (Ginsburg, unpublished), which results in a complete disintegration of cells. The fact that tumour cells exposed to hypotonicity became very sensitive to the action of trypsin whereas cells incubated in normal saline were unaffected suggests the possibility that streptococcal haemolysins

affect the permeability of tumour cells in such a way that proteolytic enzymes may penetrate into the interior of the cell, and that proteolytic enzymes which by themselves cause no apparent changes to the cell membrane attack certain substrates which have been unmasked due to the action of haemolysins.

Moreover, the cytopathological action of streptococcal proteinase on mammalian cells injured by streptococcal haemolysins has been shown to be unspecific. As neither streptococcal proteinase, nor SLS or SLD, can be inhibited by various body fluids suggest, that such synergistic effect may occur *in vivo* in foci of infections with group A haemolytic streptococci. The lack of specificity of streptococcal proteinase in this effect suggest that other proteolytic enzymes of tissue or blood origin (cathepsins, plasmin) may act in a similar manner.

These suggestions on the *in vivo* action of these agents is supported by the findings of Kellner and Robertson (1954*a*, *b*) who have shown that cardiac lesions bearing some similarity to those seen in human beings or laboratory animals infected with streptococci could be produced in rabbits by the intravenous injection of streptococcal proteinase as well as by various other proteolytic enzymes. Similarly the intravenous injection of SLS to various laboratory animals resulted in histological damage to various internal organs (Barnard and Todd, 1940). It is possible that these factors may play a role in the production of cardiac lesions and other injuries caused by haemolytic streptococci. Studies on this point are in progress.

SUMMARY

The combined action of streptococcal haemolysins (streptolysin D, streptolysin S and streptolysin O) and various proteolytic enzymes (proteinase, trypsin, papain) on Ehrlich tumour cells is described.

Tumour cells incubated with various streptococcal haemolysins swell, send out large pseudopodia and appear dead when vitally stained with trypan blue.

Various proteolytic enzymes (proteinase, trypsin, papain) which cause no apparent injury to tumour cells disintegrate cells which have been previously damaged by streptococcal haemolysins. The action of haemolysins and proteolytic enzymes is synergistic in nature.

The combined action of streptolysin O and the various proteolytic enzymes on tumour cells is completely abolished by ascitic fluid and rabbit's sera. On the other hand these body fluids do not affect the action of streptolysin D, streptolysin S and streptococcal proteinase on these cells.

The possible action of streptococcal haemolysins and proteolytic enzymes *in vivo* is discussed.

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THE EFFECT OF PREGNANCY AND OF RENIN ON THE BLOOD CONTENT OF HYPERTENSINOGEN IN RATS

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WHILE seeking an explanation for the fall in blood pressure which occurs in hypertensive rats during pregnancy it was found that pregnant rats become refractory to the pressor action of renin (Mackaness and Dodson, 1957) although they react normally to hypertensin (Dodson, 1957). Pregnancy also interferes with the diuretic and natriuretic effects of renin which are thought to be due to a direct action of hypertensin on the renal tubule and independent of any rise in blood pressure (Mackaness, 1959). These findings suggested that when renin was injected into pregnant animals it did not cause a normal release of hypertensin from its substrate (hypertensinogen) in the blood. This could occur in several ways: because of a reduced substrate concentration in the blood, by rapid elimination or destruction of renin, or by the presence of an inhibitor of the renin-hypertensin reaction.

Renin substrate is believed to be a component of the α_2-globulin fraction of the plasma proteins (Plentl, Page and Davis, 1943). It has been shown that there is a fall in the concentration of globulins in the plasma of some animal species during pregnancy (Dalgarno, Godden and McCarthy, 1950; Dunlap and Dickson, 1955). Several workers have demonstrated changes in the hypertensinogen of the blood following interference with adrenal function (Helmer and Griffiths, 1951; Page and Lewis, 1951) or the administration of various steroid hormones (Guadino, 1944; Helmer and Griffiths, 1951). Moreover, Houssay and Dexter (1942) found that the level of hypertensinogen in the blood of adrenalectomized dogs was correlated with their sensitivity to renin. In view of these findings it was necessary to compare the concentrations of hypertensinogen in the blood of pregnant and non-pregnant animals.

When renin is injected intravenously into dogs, a fall in the concentration of hypertensinogen in the plasma can be demonstrated. If pregnancy interferes with the renin-hypertensin reaction it might be expected that injection of renin would fail to lower the hypertensinogen content of the blood of pregnant rats. Having found values for the concentration of hypertensinogen in the plasma of normal and pregnant rats it was possible to extend the investigation in order to examine this possibility.

MATERIALS AND METHODS

Animals.—Normal female rats of Wistar origin weighing 150–200 g. at 12–16 weeks of age were used to obtain samples of plasma for estimation of their hypertensinogen content. Non-pregnant and late pregnant animals were anaesthetised with nembutal and bled from a cannula in the abdominal aorta after the intravenous injection of 150 I.U. of heparin.

The age of pregnancy was estimated by counting and weighing the foetuses. The average foetal weight at term was 5–6 g. The rats used for hypertensin assays were young male rats of the same strain.

Renin.—A lyophile-dried extract of pig kidney was prepared as described by Haas, Lamfrom and Goldblatt (1954). The intravenous injection of 1·5 mg. of this preparation produced an average blood pressure rise of 30 mm. Hg. in a group of 3 unanaesthetised rabbits. This amount of renin is referred to as one rabbit unit (1·0 R.U.).

Charcoal.—Animal charcoal (B.D.H.) was used for adsorption of hypertensin. It was cleaned by washing successively with glacial acetic acid, water and methanol, and was dried at 110°.

Extraction of hypertensin.—The hypertensinogen content of plasma was estimated as its hypertensin equivalent. The basis of the method was a quantitative adaptation of Peart's procedure for large scale preparation of hypertensin (Peart, 1955). Hypertensin was adsorbed on charcoal during the reaction and was eluted subsequently with glacial acetic acid. The method has the advantage that it gives a more quantitative yield because hypertensin while adsorbed on charcoal is unaffected by hypertensinase. Moreover, by preliminary adsorption of plasma with charcoal the only vasoactive material recovered in the final eluate is that released during the reaction of the plasma with renin.

Red cells were separated by centrifuging and the plasma was shaken with charcoal (7·0 mg./ml.). This charcoal was discarded after centrifugation. Plasma was then mixed with charcoal (7·0 mg./ml.) followed by the addition of an excess of renin, (2·0 R.U./ml.). The mixture was shaken mechanically for 30 min. at room temperature in glass stoppered tubes. The charcoal was deposited by centrifuging and washed by resuspending in a large volume of water. The charcoal was again separated by centrifuging and the washings were removed as completely as possible. The adsorbed products of the renin reaction were then eluted by mechanical shaking for 20 min. with three 2·0 ml. volumes of redistilled glacial acetic acid. The eluate was transferred each time to a 1 in. diameter test tube and evaporated to dryness under reduced pressure at 60°. The tube was undisturbed during evaporation and the eluate concentrated at the bottom of the tube. Each eluate was stored *in vacuo* at 5° until ready for assay, when it was dissolved in a measured volume of 0·9 per cent NaCl solution.

Hypertensin assay.—The yield of hypertensin from plasma was estimated from the pressor activity of the eluate. A four-point parallel line assay was carried out in anaesthetised rats using synthetic hypertensin as the standard (The synthetic hypertensin used was Preparation Number 19,990 a, generously supplied by Ciba Limited, Basel). The animals used for the assay were narcotised with alcohol given by stomach tube in a dose of 0·5 ml. of a 12 per cent solution of alcohol per 100 g. body weight. Alcohol was used as it caused no circulatory depression and the sensitivity of the preparation was much higher than with urethane (Dodson, 1957 ; Peart, 1955) or with nembutal. Polyethylene cannulae (Intramedic tubing was used (Clay-Adams Company, Inc. N.Y.), sizes PE 50 and PE 10) were tied into the right and left femoral veins and the carotid artery. The latter cannula was connected to a small-bore mercury manometer. Heparin (150 I.U.) was injected intravenously and the blood pressure was lowered by intravenous injection of pentolinium tartrate (May and Baker Ltd.) given over 5 min. in a total dose of 0·5 mg. A syringe containing an unknown solution and one containing the standard was attached to each femoral cannula. The concentration of the solution used was adjusted so that the volumes injected for the high and low doses were 0·08 and 0·02 ml. respectively. In the case of the standard the concentration used was invariably 1·25 μg. hypertensin per ml. For the unknown, a suitable concentration was found by trial and error. Three high and three low doses of the standard and unknown solutions were given in random order. Statistical analysis of the results was made according to Bliss and Marks (1939), and included the calculation of an average Standard Error for each estimate.

RESULTS

Recovery of added hypertensin

Peart (1955) found that the yield of hypertensin obtained when the reaction was carried out in the presence of charcoal was greater than that obtained with any of the three other methods which he tested. To show that the method was

quantitative, synthetic hypertensin was added to plasma which had been freed of hypertensin. The plasma was then taken through the whole reaction and extraction procedure. The recoveries obtained are shown in Table I.

TABLE I.—*Recoveries of Synthetic Hypertensin Added to Hypertensin-free Plasma*

Hypertensin added (μg./ml.)	Hypertensin recovered (μg./ml.)	Best estimate of recovery (per cent)
0·60	0·43–0·66*	88
0·30	0·25–0·34	97

* Fiducial limits of estimate, $P = 0{\cdot}01$.

Reproducibility of the method

The large number of assays involved made it impossible to do routine duplicate estimations. This was done, however, on one sample each of normal and pregnant plasma. The duplicate estimates obtained are set out in Table II.

TABLE II.—*Duplicate Estimations of the Hypertensinogen Content of the Plasma of a Pregnant and a Non-Pregnant rat*

Non-pregnant	Pregnant
0·69±0·05 (S.E.) μg./ml.	0·36±0·02 (S.E.) μg./ml.
0·74±0·05 (S.E.) μg./ml.	0·43±0·02 (S.E.) μg./ml.

The concentration of hypertensinogen in the plasma of normal and pregnant rats

Samples of plasma from pregnant and non-pregnant rats were treated in parallel. Although it was possible to estimate the content of hypertensinogen in the blood of a single animal, a better and more convenient estimate was obtained from a sample of pooled plasma from a group of 3–5 rats. Six pooled samples obtained from pregnant and non-pregnant rats were tested. They were obtained from 23 pregnant and 27 non-pregnant animals. The results have been set out in Table III. The difference in the concentration of hypertensinogen in the plasma of pregnant and non-pregnant rats was highly significant ($P < 0{\cdot}001$).

TABLE III.—*The Concentration of Hypertensinogen in the Plasma of Normal and Pregnant Rats Expressed as its Hypertensin Equivalent*

	Normal	Pregnant		
Test number	Hypertensinogen concentration (± S.E.) (μg./ml.)	Mean foetal weight (g.)	Mean foetal number	Hypertensinogen concentration (± S.E.) (μg./ml.)
1	0·53±0·07	5·4	9	0·22±0·02
2	0·62±0·04	5·3	9	0·22±0·03
3	0·61±0·08	5·1	11	0·27±0·06
4	0·51±0·02	5·0	9	0·29±0·02
5	0·47±0·02	5·1	9	0·15±0·01
6	0·72±0·05*	5·6	9	0·40±0·02*
Means ± S.E.M.	0·58±0·05			0·26±0·03

* Mean of duplicate estimates. "t" for differences between means = 13·5, $P < 0{\cdot}001$ (analysed as differences between pairs).

The effect of the injection of renin on the concentration of hypertensinogen in plasma

Pregnant and non-pregnant rats were narcotized with alcohol. Cannulae were inserted into a carotid artery and femoral vein and heparin (150 I.U.) was injected intravenously. The carotid blood pressure was recorded graphically. Three intravenous injections of renin (0·025 mg./100 g. body weight) were given at 10 min. intervals. Each animal was bled 10 min. after the last injection from its carotid cannula and the blood was collected in chilled tubes. The blood was kept chilled until after the charcoal and renin were added to the separated plasma. The plasma was treated in the usual way except that the preliminary adsorption with charcoal was omitted in case the plasma still contained circulating renin.

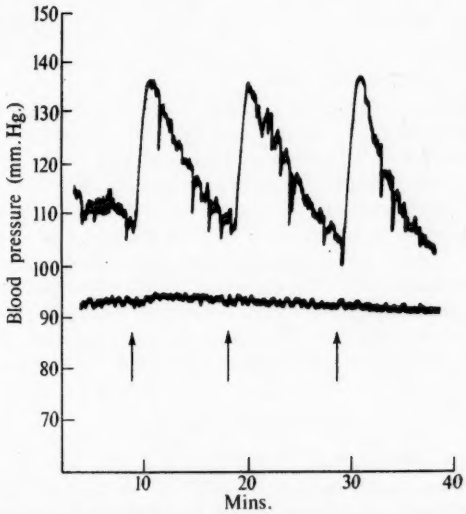

FIG. 1.—Blood pressure tracings from a normal (upper) and a pregnant (lower) rat anaesthetised with alcohol. Each was injected intravenously with three doses of renin (0·025 mg./100 g. body weight) at the arrows.

Tests were made on 6 normal and 6 pregnant rats. The results are shown in Table IV. A clot developed in the reaction mixture of pregnant rat number 6 (perhaps because heparin was not injected) and a result was not obtained for this animal. Typical of the blood pressure responses obtained are those shown in Fig. 1. Normal animals consistently gave responses of 30–40 mm. Hg. Pregnant animals on the other hand showed little or no change in blood pressure.

When the content of hypertensinogen in the plasma after injection of renin was compared with that found in uninjected animals it was seen that a fall in substrate levels had occurred. The falls of 62 and 55 per cent respectively in pregnant and normal animals were statistically significant. It was clear, therefore, that the insensitivity to renin in pregnant animals could not have been due to inhibition of the renin-hypertensin reaction or to premature destruction of renin *in vivo*.

TABLE IV.—*The Concentration of Hypertensinogen in the Plasma of Pregnant and Non-Pregnant Rats Following Intravenous Injection of Renin*

Non-pregnant		Pregnant	
Test number	Hypertensinogen content (μg./ml.)	Test number	Hypertensinogen content (μg./ml.)
1	0·28±0·05	1	0·22±0·01
2	0·34±0·06	2	0·03±0·004
3	0·32±0·03	3	0·01±0·001
4	0·15±0·01	4	0·11±0·005
5	0·21±0·01	5	0·11±0·005
6	0·28±0·01	6	Lost
Means ± S.E.M.	0·26±0·03		0·10±0·04

Pressor response to a large dose of renin

Four normal and four pregnant animals were narcotized with alcohol and cannulae were inserted into a carotid artery and a femoral vein. The blood pressure was lowered with pentolinium tartrate and when the blood pressure was steady an intravenous injection of renin was given. The dose injected (2·5 mg./100 g. body weight) was 100 times that used in the previous experiment. The dose was repeated at the end of 1 hr. The responses shown in Fig. 2 are typical; they were chosen for illustration because the 2 rats had similar basal blood pressures. Although the response was smaller and of shorter duration in the pregnant animal the shape of the 2 curves is very similar. Apparently pregnant rats differ only quantitatively in their response to renin and these differences in response could be accounted for by differences in the amount of substrate available.

DISCUSSION

It has been shown (Mackaness, 1959) that not only the pressor, but also the sodium excreting (natriuretic) action of renin is depressed by pregnancy. The natriuretic action of renin is thought to be a direct effect of hypertensin on the renal tubule and to be independent of the vascular response. In view of this it would appear that pregnancy interferes with the renin-hypertensin reaction rather than with vascular reactivity. The present observations have shown that when renin is injected into normal and pregnant animals the concentration of substrate in the plasma is reduced. It thus appears that the dose of renin used caused release of hypertensin in pregnant rats without at the same time producing any significant rise in blood pressure. Dodson (1957) has shown that pregnant rats respond normally to hypertensin; and the experiment illustrated in Fig. 2 shows that there is nothing qualitatively different about the renin response of pregnant animals. It seems likely, therefore, that the rate of hypertensin release by normal doses of renin is too slow to produce a rise in blood pressure in pregnant rats. However, it cannot be said whether the concentration of hypertensinogen found in the blood of pregnant rats is low enough to affect significantly the rate of the renin-hypertensin reaction. Taquini, Braun-Menéndez, Fasciolo, Leloir and Muñoz (1943) found that a 2-fold variation in substrate concentration did not influence the velocity of the renin-hypertensin reaction *in vitro*. They assumed that the enzyme was fully active. Plentl and Page (1943), however, claimed that

the reaction velocity was proportional to substrate concentration. Quite apart from this contradiction, evidence obtained from studies *in vitro* cannot go far towards predicting behaviour *in vivo*, where the enzyme and the products of the reaction are both being actively destroyed, and substrate is being regenerated at an unknown rate during the course of the reaction.

Several studies *in vivo* on the other hand, suggest that variations in hypertensinogen concentration may be significant. Houssay and Dexter (1942) have shown that adrenalectomy causes a fall in the level of hypertensinogen in the

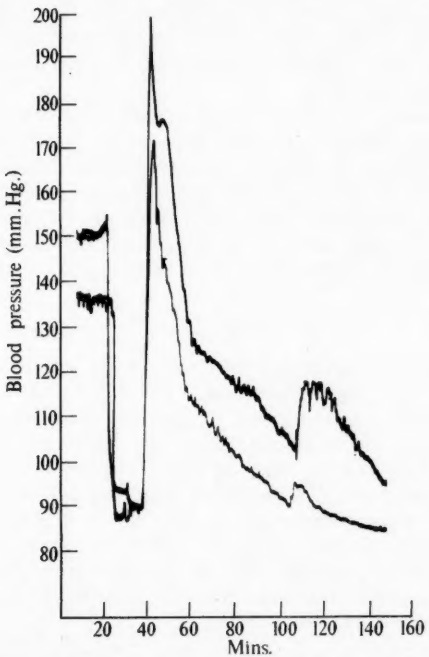

FIG. 2.—Blood pressure tracings of a normal (upper) and a pregnant (lower) rat. The initial fall in blood pressure was produced by pentolinium tartrate (0·5 mg.). Injections of renin (2·5 mg./100 g. body weight) at 40 min. and at approximately 100 min.

plasma ; and that the extent of the fall is correlated with the loss of sensitivity to renin. The reduction in hypertensinogen concentration in plasma following the injection of renin has been thought to account in part for the diminished renin response given by animals made tachyphylactic to renin (Page, 1939 ; Page and Helmer 1940 ; Houssay, Braun-Menéndez and Dexter, 1942 ; Goldblatt, Lamfrom and Haas, 1953). Conversely a large number of observations have shown that the hypertensinogen content of plasma rises following nephrectomy and is associated with an increased sensitivity to renin (Houssay and Dexter, 1942 ; McCubbin and Page, 1954 ; Collins and Harakal, 1954). It is apparent, therefore, that sensitivity to renin is altered by variations in plasma hypertensinogen

concentration, but quantitative data to show the importance of substrate concentration on sensitivity to renin are lacking.

The reason for the fall in the concentration of hypertensinogen in the plasma during pregnancy is obscure. The blood volume of pregnant rats is increased by about 40 per cent near term (Bond, 1948), but this probably does not explain the fall in concentration of hypertensinogen. It may be caused by hormonal changes, since adrenalectomy produces a fall in blood hypertensinogen which can be restored by the administration of various hormones (Helmer and Griffiths, 1951; Page and Lewis, 1951). Moreover, administration of adrenal cortical hormones (Guadino, 1944), oestrogens (Helmer and Griffiths, 1951) or ACTH (Haynes, Forsham and Hume, 1953) to normal animals causes an increase in the concentration of hypertensinogen in the blood. The sensitivity to renin of animals so treated has not been explored.

Although the present experiments suggest that a lowered substrate concentration in the blood may account for the diminished sensitivity to renin during pregnancy, there is still no evidence of a relationship between the fall in renin substrate concentration in the blood and the disappearance of renal hypertension during pregnancy. An approach to this question might be possible by studying the effect of raising the substrate level in the blood by injecting exogenous hypertensinogen into hypertensive rats in whom the blood pressure has fallen as a result of pregnancy.

SUMMARY

The concentration of hypertensinogen has been measured in the blood of normal and pregnant rats. In late pregnancy the blood level fell significantly from the normal value of $0{\cdot}58 \pm 0{\cdot}05$ μg./ml. hypertensin equivalent per ml. of plasma to $0{\cdot}26 \pm 0{\cdot}03$ μg./ml. Reasons are given for believing that the diminished response of pregnant rats to renin may be due to the low levels of hypertensinogen in the blood. The intravenous injection of renin in amounts sufficient to cause blood pressure responses in non-pregnant but not in pregnant rats, produced a comparable fall in substrate level in both, indicating that the renin-hypertensin reaction is not inhibited in pregnant rats. It has not been shown that the reduction in renin substrate concentration is the reason for the disappearance of hypertension in renally ischaemic rats during pregnancy.

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STUDIES ON THE ANTIGENIC STRUCTURE OF VIRULENT AND NONVIRULENT BRUCELLAE WITH THE AID OF THE AGAR GEL PRECIPITATION TECHNIQUE*

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OLITZKI AND SULITZEANU (1957, 1958) examined the antigen-antibody reaction between *Brucella suis* antisera and their corresponding antibodies by the Ouchterlony (1948) technique and noted the appearance of at least 6 precipitation lines.

Further experiments of Olitzki and Sulitzeanu (1958) distinguished the different antigens of *Br. suis* by chemical and physical procedures and determined the conditions under which a maximal number of precipitation lines in agar could be produced. Sulitzeanu (1958) showed that no one of the precipitation lines could be correlated with the presence or absence of agglutinins in the immune serum. He found, however, a good correlation between agglutinating and passive protection power of sera. For all these experiments *Br. suis* was chosen because of the ease with which strongly precipitating antisera could be obtained against it.

The general plan of the present work was as follows: rabbits were immunized with antigens derived from different strains of the genus *Brucella*. These antisera were tested for their precipitating power against homologous and heterologous antigens. Finally, quantitative titrations similar to those employed by Feinberg (1956) and absorption tests were carried out, in order to determine whether there exist differences in the antigenic structures of *Br. abortus*, *Br. melitensis* and *Br. suis*.

MATERIAL AND METHODS

The experimental procedures have been described in the earlier papers of Olitzki and Sulitzeanu (1957, 1958) and Sulitzeanu (1958). Certain modifications of the techniques and a few additional details are given below:

Strains

Br. melitensis, strain 5, *Br. suis*, strain 39, and *Br. abortus*, strains 2308 and 19 were employed by Olitzki (1953) in experimental infections of mice with the aid of the mucin technique and subcultured for 6 years on trypticase soy agar. The vaccine strain Rev. I of *Br. melitensis* was isolated by Herzberg and Elberg (1955) and employed as vaccine strain by Elberg and Faunce (1957), while the virulent strain 6015 of *Br. melitensis* was used by them as challenge. The strain designated as Ru is identical with that employed by Vershilova (1954) and Vershilova and Kokorin (1954) in immunization of individuals exposed to brucellosis infections. Another *Br. abortus*, strain 19, was kindly provided by Dr. H. S. Cameron, School of Veterinary Medicine, University of California, Davis, California, and was designated as strain 19 Da.

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Preparation of antigens for immunization and preparation of immune sera

The antigens were prepared by the same method as those of *Br. suis* described by Olitzki and Sulitzeanu (1958). For the preparation of immune sera a mixture was prepared which contained 7·65 ml. Kleerol, 1·35 ml. Arlacel A, 9·0 ml. physiological saline and 180 mg. acetone-killed and dried bacteria. The adjuvant mixture contained 10 mg. of bacteria per ml. Groups of rabbits received subcutaneously 4 doses of 1·0 ml. and then 4 doses of 2·0 ml. at weekly intervals. Between the fourth and the fifth injection a 2 week interval was left and a preliminary examination of the serum carried out. Blood was obtained by cardiac puncture 2 weeks after the eighth injection.

Antigens for gel precipitation

The bacteria were repeatedly disintegrated in a disintegrator (Mickle, Mill Works, Gomshall, Surrey). Since the experiments of Sulitzeanu (1958) showed that the insoluble sediments do not produce additional lines, no further attempts to remove them by centrifugation were made.

Agar for gel precipitation

Difco Bacto-Agar was used through all experiments. Since preliminary experiments showed that at lower than physiological salt concentrations sharper precipitation lines appear, the following quantities of salts were added to 1 litre of 1 per cent agar: 0·353 g. NaH_2PO_4, 0·639 g. Na_2HPO_4 and 0·172 g. NaCl. The resulting pH was 7·2. Since each plate contained only 7·0 ml. of agar and 0·7 ml. of antigens and antibodies in physiological saline distributed in seven holes, this low salt concentration of the agar proved to be favourable for the development of the precipitation lines.

Diffusion test

In order to assure a constant reproduction of the results, all experiments were carried out on small Petri dishes with a diameter of 5·0 cm. The holes for the antigens and sera were made with the aid of cutters kindly provided by Dr. C. Lamanna, Scientific Director, Naval Biological Laboratory, Oakland. All the figures attached to this paper show in 2-fold magnification the position of the holes, which was never changed during the entire work. This method, introduced by Feinberg (1956) also had the advantage that volumes no greater than 0·1 ml. per hole could be employed. Since the diffusion of the reagents from the peripheric holes was practically possible only in one direction from the periphery to the centre, the reaction lines appeared quickly and the first results were observed after an incubation of 20 hr. The plates were incubated in plastic bags closed with rubber bands. Cotton-wool moistened with water served to prevent drying.

RESULTS

In the first 3 experiments an undiluted immune serum was placed in the central hole and undiluted bacterial antigens around it. The results of these experiments are demonstrated in Figs. 1, 2 and 3. In all these figures a diffuse precipitation zone appears around the serum source, and was designated as D. This zone was then followed by 6 sharp lines, which are easily visible in Figs. 1 and 2 opposite the *Br. suis* antigen. Generally, the thickness of these lines decreased from the centrum to the periphery. Line 6, nearest to the central serum source, was the thickest, and lines 1 and 2, nearest to the peripheric antigen sources, were the weakest and were not regularly produced with each antigen. The only exception was the appearance of an extremely strong line near the antigen source of *Br. abortus*, strain 19. This crescent-shaped line around the antigen sources A is well visible in Figs. 1 and 3. Those lines situated nearest to the serum source, such as lines 4, 5 and 6, were produced by all strains. It seems, therefore, that they were produced by non-specific antigens. On the

other hand, the several crossings of the lines (*e.g.* in Fig. 1 between antigen sources M and A and in Fig. 2 between M and A, M and S and between S and A) indicated that there existed quantitative differences of the antigenic pattern.

Since these experiments with undiluted sera and antigens did not permit final conclusions, further experiments were carried out, where antigens were placed around the central hole containing diluted antiserum. An experiment of this kind is reproduced in Fig. 4, where the central hole contains *Br. suis* antiserum diluted 1/3. The lines 1, 2 and 3 have completely disappeared. There remained only the diffuse precipitation zone around the serum source, the thick line 6, the sharp and thin line 5 and the more thick and diffuse line 4. The 2 latter appeared opposite *Br. melitensis* and *Br. suis* and disappeared opposite *Br. abortus* strain 19, while line 6 was common for all the 3 antigens. On the other hand, there appeared again the sharp crescent-shaped precipitation line near the source of *Br. abortus* 19, which was already shown in Figs. 1 and 3. In order

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EXPLANATION OF PLATES

FIG. 1.—Diffusion pattern of three antigens reacting with undiluted *Br. suis* antiserum. I.S. Suis = *Br. suis* immune serum. A = *Br. abortus* 19; M = *Br. melitensis* 6015; S = *Br. suis* 39.

FIG. 2.—Diffusion pattern of three antigens reacting with undiluted *Br. melitensis* antiserum. I.S. MEL. = *Br. melitensis*, strain 5, immune serum. Antigens the same as in Fig. 1.

FIG. 3.—Diffusion pattern of three antigens reacting with undiluted *Br. melitensis* antiserum. I.S. REV. I = *Br. melitensis*, strain Rev. I, immune serum. Antigens as in Figs. 1 and 2.

FIG. 4.—Diffusion pattern of three antigens reacting with *Br. suis* antiserum, diluted 1 : 3. Abbreviations as in Fig. 1.

FIG. 5.—Varying amounts (mg.) of *Br. melitensis*, strain 6015, antigen reacting with *Br. melitensis*, strain 5, immune serum.

FIG. 6.—Varying amounts (mg.) of *Br. abortus*, strain 2308, antigen reacting with *Br. suis* antiserum.

FIG. 7.—Varying amounts (mg.) of *Br. melitensis*, strain Rev. I, antigen, reacting with *Br. melitensis*, strain 5, antiserum.

FIG. 8.—Varying amounts (mg.) of *Br. melitensis*, strain Rev. I, antigen reacting with its homologous antiserum.

FIG. 9.—Minimal amounts (μg.) of *Br. melitensis*, strain 6015, and strain Rev. I antigens reacting with *Br. suis* immune serum. Rev = non-virulent strain Rev. I; Vi = virulent strain 6015.

FIG. 10.—Minimal amounts (μg.) of *Br. melitensis*, strain 6015, and *Br. suis*, strain 39, antigen reacting with *Br. suis* immune serum. Abbreviations as in Fig. 1.

FIG. 11.—Varying amounts (mg.) of the vaccine strain Ru reacting with *Br. melitensis* strain Rev. I antiserum.

FIG. 12.—Varying amounts (μg.) starting from 625 μg. of *Br. melitensis*, strain 6015, antigen reacting with its homologous antiserum.

FIG. 13.—Varying amounts (μg.) starting from 625 μg. of *Br. melitensis*, strain 6015, antigen reacting with *Br. melitensis*, strain Rev. I, antiserum.

FIG. 14.—Varying amounts (μg.) starting from 1250 μg. of *Br. melitensis*, strain Rev. I, antigen reacting with *Br. melitensis*, strain 5, antiserum.

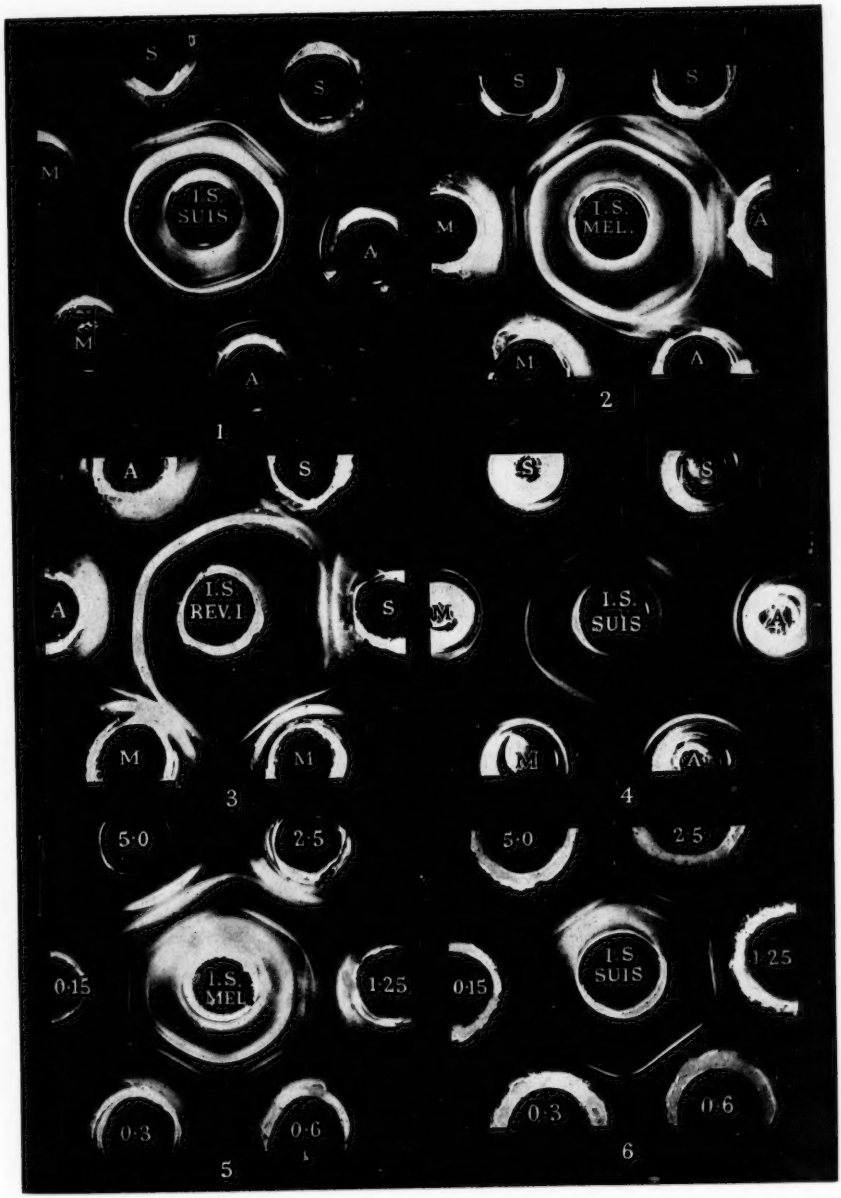
FIG. 15.—Varying amounts (μg.) starting from 1250 μg. of *Br. melitensis*, Strain Rev. I, antigen reacting wilh its homologous antiserum.

FIG. 16.—*Br. melitensis*, strain 6015, antigen reacting with varying dilutions of its homologous immune serum. AGMEL = antigen of *Br. melitensis*; I.S. = homologous immune serum.

FIG. 17.—*Br. abortus*, strain 19, antigen reacting with varying dilutions of its homologous immune serum. AG19 = antigen of *B. abortus*, strain 19. IS = homologous immune serum.

FIG. 18.—*Br. suis* antiserum reacting with antigen of *Br. melitensis*, strain 6015, *Br. abortus*, strains 19 Je. and 19 Da.

FIG. 19.—*Br. suis* antiserum antisera reacting with antigen *Br. abortus*, strains 2308, 19 Je. and 19 Da.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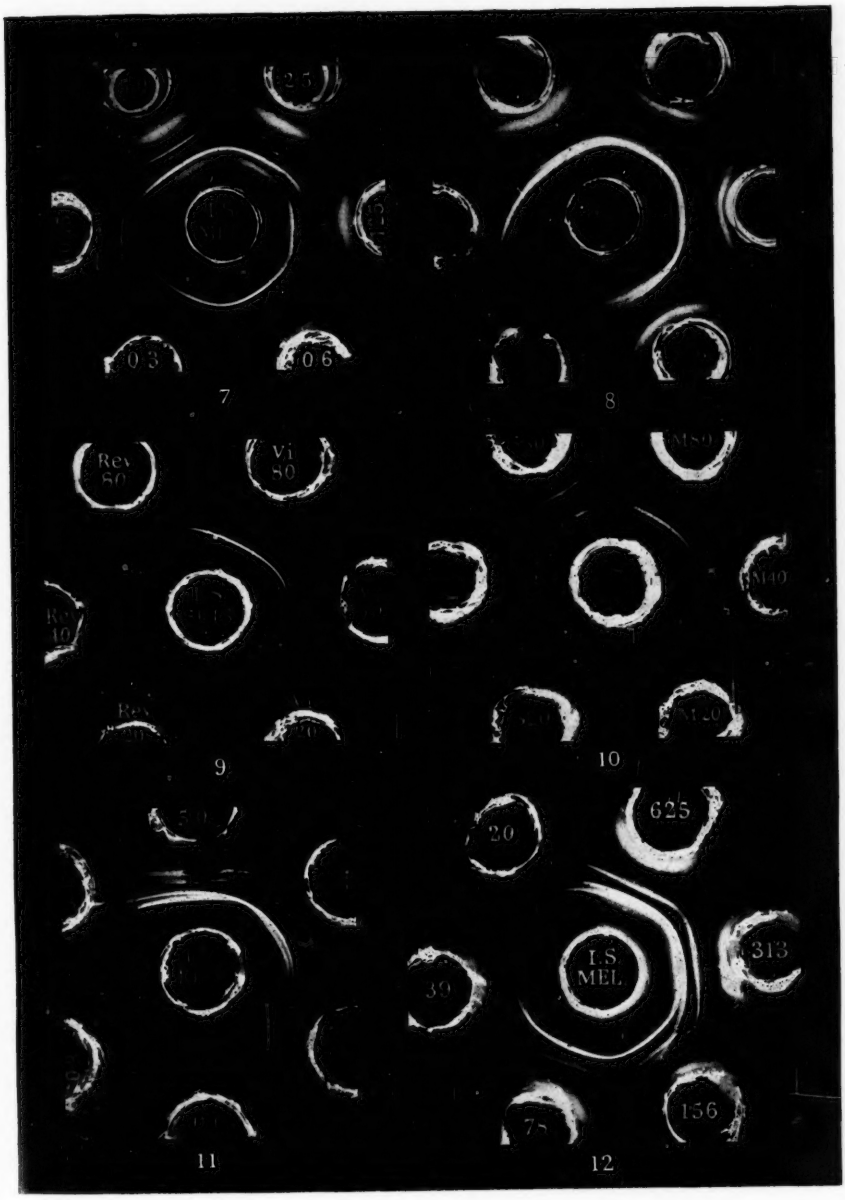
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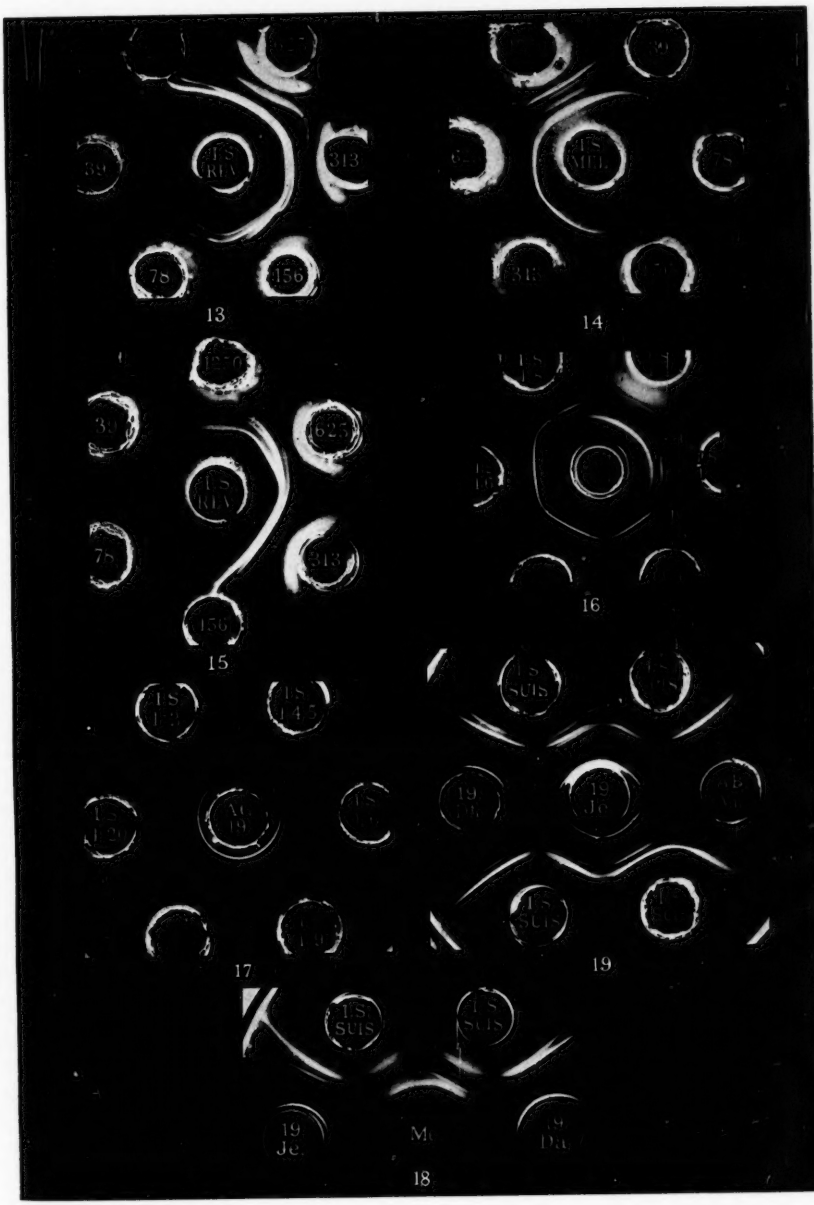

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to determine whether these and similar diffusion patterns were the results of quantitative or qualitative differences in the antigenic structures, the following experiments were carried out with varying amounts of bacterial antigen placed around a constant amount of undiluted antiserum.

A typical experiment is shown in Fig. 5. Around the central source of 0·1 ml. antiserum varying quantities of *Br. melitensis* strain 6015 antigen were placed. The figure shows that the diffuse zone D, and the lines 6, 5 and 4 still persisted at 0·15 mg. Line 6 became split up in 2 partial lines. Other lines, as the crescent-shaped line 1 appeared only in the vicinity of at least 0·3 mg. and line 2 at 2·5 mg. antigen. Another experiment of this kind is presented in Fig. 6, where varying quantities of *Br. abortus*, strain 2308, antigen had reacted with *Br. suis* antiserum. The lines 6, 5, 4 and 3 appeared in a range from 5·0–0·15 mg. antigen. However, opposite the hole containing 0·3 mg. of antigen the lines were more distinct than opposite all the other holes containing varying amounts of antigen.

While the virulent strains *Br. melitensis* 6015 and *Br. abortus* 2308 produced line 6 even in the presence of 0·15 mg. of antigen, the vaccine strain *Br. melitensis*, Rev. I, showed a somewhat weaker precipitating potency. Fig. 7 shows that with *Br. melitensis* immune serum line 6 appeared still opposite 0·15 mg. of antigen, while according to Fig. 8 the reaction almost disappears at 0·3 mg. of antigen. All the other reactions appeared in the presence of great amounts of antigen ranging from 5·0 to 1·25 mg. with the exception of the line 1, which in the reaction with the strain 5 antiserum was still visible in the presence of 0·15 mg. and with the Rev. I antiserum in the presence of 0·6 mg. of antigen.

In order to determine the minimal precipitating quantities of the virulent strains, smallest quantities of the antigens were brought in contact with the corresponding antisera. Fig. 9 and 10 show the results of 2 titration experiments. In Fig. 9 the precipitating potency of the virulent strain *Br. melitensis* is compared with that of the nonvirulent strain Rev. I towards a *Br. suis* antiserum. While the antigen of the virulent strain still reacted when only 20 μg. of bacterial substance are present, the reaction of Rev. I antigen did not appear, although 80 μg. were present. In Fig. 10 the precipitating activities of *Br. melitensis*, strain 6015, and *Br. suis* antigen were compared. The lines 6 of both strains appeared in the presence of 20 μg., while line 5 of *Br. suis* still appeared in the presence of 80 μg. of antigen.

Relatively small amounts of bacterial substance of the vaccine strain Ru. reacted with *Br. melitensis* (5) antiserum while with *Br. suis* and *Br. melitensis* (Rev. I) antiserum positive reactions were observed in the presence of at least 625 μg. of bacterial substance as demonstrated in Fig. 11.

In order to determine whether the line which still appears at minimal antigen concentrations is identical with line 6, experiments were started with maximal amounts of 625 and 1250 μg. of bacterial substance, decreasing to 39 and 20 μg. respectively. These experiments are summarized in Fig. 12–15. Fig. 12 and 13 show the reactions of *Br. melitensis*, strain 6015, in 2 different antisera. In both of these all lines have disappeared at 39 μg. of bacterial substance with the exception of line 6 which reached 20 μg. in Fig. 12 and 39 μg. in Fig. 13.

Fig. 14 and 15 show corresponding experiments with strain Rev. I. Line 6 in both figures reached the limit of 156 μg. and in Fig. 14 traces of a diffuse line are still visible at 78 μg.

The result of these and other experiments, which were not presented in photo-

graphs, are summarized in Table I. The table shows that lines 5, 6 and D were produced by all strains. There existed only quantitative differences between the different strains. The virulent *Br. melitensis*, strain 6015, produced these lines with minimal quantities of bacterial substance, while from other strains greater amounts had to be present. Lines 2, 3 and 4 were rarely produced by the vaccine strain *Br. abortus* 19. This strain, however, produced regularly a strong line 1 near to its antigen source.

TABLE I.– *The Minimal Amounts of Disintegrated and Dried Bacterial Substance Required for the Production of Precipitation Lines in the Presence of* 0·1 *ml. of Undiluted Immune Serum*

	Antigen.		Minimal amount of dried bacterial substance (μg.) which produced precipitation lines.						
Antiserum.	Species.	Strain.	1	2	3	4	5	6	D
Br. suis (39)	*Br. melitensis*	6015	—	5000	1250	313	156	20	78
		Rev. I	—	—	2500	1250	625	156	156
		5	—	5000	1250	625	156	78	156
	Br. suis	39	—	—	—	1250	78	20	78
	Br. abortus	2308	—	—	313	156	156	156	156
		19	1250	—	—	5000	1250	156	156
		Ru.	2500	2500	2500	1250	1250	625	625
Br. melitensis (5)	*Br. melitensis*	6015	313	2500	156	156	156	20	78
		Rev. I	156	2500	1250	1250	625	156	156
		5	2500	2500	1250	625	313	156	156
	Br. suis	39	—	2500	313	625	313	20	156
	Br. abortus	2308	—	—	313	313	156	156	78
		19	2500	—	—	—	2500	313	313
		Ru.	—	2500	1250	313	313	78	78
Br. melitensis (Rev. I)	*Br. melitensis*	6015	—	313	313	156	78	20	20
		Rev. I	625	2500	2500	313	313	156	156
		5	—	—	5000	5000	2500	156	156
	Br. suis	39	—	—	—	2500	313	156	156
	Br. abortus	2308	—	5000	625	625	625	156	156
		19	2500	—	—	—	313	313	313
		Ru.	2500	1250	1250	1250	1250	625	625
Br. abortus (19)	*Br. melitensis*	6015	—	1250	1250	313	78	39	78
		Rev. I	—	—	2500	1250	625	78	78
		5	—	1250	625	625	625	10	78
	Br. suis	39	—	—	2500	625	625	78	78
	Br. abortus	2308	—	2500	2500	1250	313	10	78
		19	625	2500	2500	625	78	78	78
		Ru.	—	1250	156	156	78	78	78
Br. abortus (2308)	*Br. melitensis*	6015	—	—	2500	625	156	78	78
		Rev. I	—	—	2500	1250	313	156	156
		5	—	—	—	1250	1250	156	156
	Br. suis	39	—	—	5000	625	625	156	78
	Br. abortus	2308	—	—	2500	625	156	78	78
		19	1250	—	—	625	625	156	156
		Ru.	—	—	625	625	156	78	78

In the following experiments the antigens were placed in the centre of the plate and reacted with varying serum dilutions placed in the peripheric holes. Fig. 16 presents such an experiment, in which *Br. melitensis* antiserum reacts with the antigen of strain 6015. According to the reversion of the experimental

conditions the lines D and 6 were nearest the peripheric holes, followed by lines 5 and 4. The experiment shows that lines 5 and 4 were still visible opposite the serum dilution 1 : 8, while lines D and 6 already disappeared at the serum dilution 1 : 3. The majority of the strains reacting with different immune sera showed a similar diffusion pattern. Only a few lines were produced in the presence of diluted immune serum, mainly lines 4, 5 and 6. The highest serum titres were generally obtained with line 5. The only strain which showed another diffusion pattern was *Br. abortus*, strain 19. Lines 4, 5 and 6 never appeared in the presence of diluted immune serum. Instead, line 1 appeared around the antigen source and was still visible up to a serum dilution of 1/20, as shown in Fig. 17.

In order to prove that this antigen line of *Br. abortus* 19 was really different from the other lines which appeared at higher serum dilutions, we placed antigen of *Br. abortus* 2308 in one sector, the antigen of strain 19 in a second, and a mixture of both antigens in a third sector around the source of diluted Rev. I antiserum which yielded the highest titres of lines 4 and 5. The result showed that line 1 of strain 19 appeared everywhere, where its antigen was present, as an additional line, and never as a continuation of another antigen line. In order to ascertain that this peculiar antigen line of strain 19 is common to strains 19 of different origin, the Jerusalem strain 19 was compared with the Davis strain 19. Fig. 18 shows the results of such an experiment. As immune serum a *Br. suis* antiserum was used, which produced all 6 lines regularly. In the centre of the 3 antigen sources was placed *Br. melitensis*, strain 6015, on the right side was strain 19 Da. and on the left side was strain 19 Je. The figure shows that the antigens of strain 19 Je. and 19 Da. also produced the sharp line 1, while *Br. melitensis*, which produced all 6 lines showed a more diffuse, bride line which was situated in a wider distance from its antigen source and did not constitute a continuation of lines 1 of the 2 strains 19. Fig. 20 shows a similar arrangement of both strain 19 antigens. In the 3 central holes 3 different *Br. abortus* antigens were placed : the non-virulent strains 19 Je and 19 Da. and the virulent strain 2308. These antigens reacted with 2 weak *Br. suis* antisera, one producing lines 4, 5 and 6, and the other only lines 5 and 6. Nevertheless, there appeared around the serum sources of both vaccine strains the additional line 1, which did not appear around the serum source of the virulent strain. The results of all these experiments are summarized in Table II. The table shows that the only lines which appeared at higher serum dilutions were lines 4 and 5, with the exception of strain 19 which produced line 1, while the titres for line 6 generally were lower. The height of the titres for line 5 depended completely on the antiserum. Two *Br. melitensis* antisera precipitated all antigens at dilutions ranging from 1/13·5 up to 1/35·0, a *Br. suis* antiserum at dilutions from 1/4·5 up to 1/20, while a *Br. abortus* antiserum yielded titres from 1/3 up to 1/6.

Absorption experiments

In the following experiments graded dilutions of an antiserum were absorbed with graded amounts of homologous and heterologous antigens and the lines produced by the absorbed antisera compared with those produced by the original antiserum. Table III shows the results obtained with a *Br. melitensis* antiserum. It shows that after treatment with small amounts of antigens the serum lost the property of producing lines 1, 2 and 3 and mostly also line 4. The only lines which still persisted were lines 5 and 6. There were no significant differences in

TABLE II.—*The Titres of Different Precipitating Immune Sera*

Immune serum.	Antigen. Species.	Strain.	Reciprocals of final dilutions producing lines. 1	4	5	6
Br. melitensis (5)	*Br. melitensis*	6015	—	20·0	13·5	4·5
		Rev. I	—	20·0	13·5	4·5
		5	—	20·0	20·0	6·0
	Br. suis	39	—	20·0	13·5	4·5
	Br. abortus	2308	—	20·0	30·0	6·0
		19	20·0	—	—	—
		Ru.	—	20·0	25·0	6·0
Br. melitensis (Rev. I)	*Br. melitensis*	6015	—	20·0	20·0	6·0
		Rev. I	—	20·0	25·0	6·0
		5	—	20·0	20·0	4·5
	Br. suis	39	—	20·0	20·0	4·5
	Br. abortus	2308	—	20·0	35·0	6·0
		19	20·0	—	—	—
		Ru.	—	20·0	35·0	4·5
Br. suis (39)	*Br. melitensis*	6015	—	13·5	13·5	3·0
		Rev. I	—	—	20·0	4·5
		5	—	13·5	20·0	4·5
	Br. suis	39	—	—	9·0	9·0
	Br. abortus	2308	—	3·0	4·5	3·0
		19	20·0	—	—	—
		Ru.	—	20·0	6·0	3·0
Br. abortus (19)	*Br. melitensis*	6015	—	—	9·0	—
		Rev. I	—	—	6·0	—
		5	—	—	3·0	—
	Br. suis	39	—	—	3·0	—
	Br. abortus	2308	—	—	4·5	—
		19	20·0	—	—	—
		Ru.	—	—	4·5	—

the combining power of the different antigens. At a serum dilution of 1/4 and an antigen concentration of 2·4 mg. per ml., *Br. melitensis*, *Br. suis* and *Br. abortus* were able to remove all precipitins. These results proved that the antigens involved in the above described precipitation reactions were shared by all strains of the genus *Brucella*, and that only quantitative differences between the strains existed.

DISCUSSION

The experiments described above showed that the same soluble antigens of *Br. suis*, which were revealed by Olitzki and Sulitzeanu (1957) by the Ouchterlony (1948) technique, are present in all strains of the genus *Brucella*. However, in order to produce all the 6 precipitation lines, a long immunization of rabbits with the aid of adjuvants was necessary. There existed certain rules in the appearance of the precipitation lines, when quantitative methods were applied. If graded quantities of antigens were titrated in the presence of a constant amount of undiluted immune serum, then smallest amounts of antigen were sufficient for the production of the lines nearest to the source of serum, mainly lines D and 6. For the production of all the other lines higher quantities of bacterial extracts were required. The regularity of the appearance of the lines decreased with their

TABLE III.—*Absorption Experiments with a* Br. melitensis *Antiserum*

Serum diluted.	Absorption with antigen. Strain.	mg. per ml.	Remaining precipitation lines with *Br. abortus.*	*Br. suis.*	*Br. melitensis.*
1/2	*Br. melitensis* (6015)	1·2	6	6	6
		0·6	6	6	6
		0·3	6	6	5, 6
		0·15	6	6	5, 6
1/4		2·4	—	—	—
		1·2	—	—	6
		0·6	—	—	6
		0·3	—	6	5, 6
		0·15	—	6	5, 6
1/2	*Br. suis* (39)	2·4	—	6	5, 6
		1·2	5, 6	5, 6	4, 5, 6
		0·6	4, 5, 6	5, 6	4, 5, 6
1/4		2·4	—	—	—
		1·2	—	—	6
		0·6	—	—	6
		0·3	—	6	5, 6
		0·15	6	6	5, 6
1/2	*Br. abortus* (2308)	2·4	—	6	5, 6
		1·2	5, 6	5, 6	5, 6
		0·6	5, 6	5, 6	5, 6
1/4		2·4	—	—	—
		1·2	—	—	6
		0·6	—	—	6
		0·3	—	6	4, 5, 6
		0·15	6	6	4, 5, 6

distance from the serum source. The only exception was the line 1 of *Br. abortus*, strain 19, which appeared with a great regularity in a great distance from the serum source just on the edge of the antigen source. There existed also quantitative differences in the ability of the different strains to produce the most common lines. Line 6 was produced by the virulent strains *Br. melitensis* (6015) and *Br. suis* (39) with a great regularity in the presence of a few μg. of bacterial substance, while the different vaccine strains required generally higher quantities. However, differences from serum to serum occurred. *Br. abortus* antigens required in their homologous immune sera generally somewhat lower quantities for the production of line 6 than in *Br. melitensis* and *Br. suis* antisera.

If a constant amount of antigen was reacting with graded quanties of antiserum, then the highest titres were obtained with line 5, which in certain cases appeared even at serum dilutions of 1/35. These results obtained with 2 different methods, one using decreasing amounts of antigen, and the other one decreasing concentrations of antiserum, show that the relative amount of a single antigen in an antigen mixture is not always correlated with its precipitinogenic potency. Antigen 6 which seems to be the main antigen of the bacterial extracts did not produce serum titres as high as those produced by antigen 5. The same observation was made with the antigen 1 of strain 19. Although high concentration of bacterial substance was required in order to make its line visible, it appeared in serum dilutions as high as 1/20 proving its relatively high potency as precipitinogen. This antigen produced antibodies even in antisera against strains, which themselves were unable to give rise to the appearance of the line, when

used as antigens in the precipitation test. However, the appearance of its specific antibody in all antisera produced in the course of this investigation proves its high potency as precipitinogenic substance.

The absorption tests showed that there existed only quantitative differences in the presence of the different antigens in all strains of the group. The most persistent antibodies against the antigens 5 and 6 were finally removed under favourable conditions, *i.e.*, a serum dilution of 1/4 and an amount of absorbing antigen in a concentration of at least 2·5 mg. per ml. independent from its origin.

There remains only to clear up the relationship of the antigens described above with those described by Braun, Burrous and Phillips (1957) and Phillips, Braun and Plescia (1958), which were characterized as DNA-proteins and gave also several precipitation lines. Experiments are now in progress, to compare them with the antigens described above.

SUMMARY

Br. abortus, *Br. melitensis* and *Br. suis* possess at least 6 soluble antigens, which could be demonstrated by the aid of the agar gel precipitation technique. These antigens differed in their relative concentration in bacterial extracts of different origins and in their ability to produce antibody titres in immune sera. No antigen specific for a single species was demonstrated among these antigens.

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THE MECHANISM OF DEVELOPMENT OF RESISTANCE TO LYSOZYME BY SOME GRAM-POSITIVE BACTERIA AND ITS RESULTS

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THE mechanism by which micro-organisms become resistant to the action of drugs is uncertain in the majority of instances. A notable exception is the production of the enzyme penicillinase by certain penicillin resistant bacteria. One of the major difficulties in the investigation of the problem of bacterial resistance is that the site and mode of action of many of the commonly used antibiotics is unknown.

"Lysozyme" is the name given by Fleming (1922) to a group of closely related enzymes, found in various tissues and secretions, which have the ability to lyse the cells of a number of bacterial species. Lysozyme acts upon susceptible bacteria by combining with and breaking down a mucopolysaccharide (Hallauer, 1929; Meyer, Palmer, Thompson and Khorazo, 1936; Epstein and Chain, 1940). This mucopolysaccharide, which is the lysozyme substrate, has been shown to be situated in the bacterial cell wall (Salton, 1952) and can be characterised chemically. Following the action of lysozyme N-acetylamino sugars and reducing groups are liberated (Epstein and Chain 1940; Salton, 1956). Thus in the lysozymes we have an antibacterial agent for which the substrate is known and can be estimated quantitatively. It was thought, therefore, to be of interest to produce resistant strains of an organism *Micrococcus lysodeikticus*, which is normally highly sensitive to lysozyme, and to carry out quantitative analysis of the walls of sensitive and resistant strains, and to see if they differed from each other.

At the start of the investigation it was considered that resistance could be due to any of the following possibilities. (1) Production by the organism of a specific lysozyme antagonist. (2) Decrease or disappearance of lysozyme substrate thus removing the site of lysozyme action. (3) Increase in lysozyme substrate to such an extent as to diminish the effect of lysozyme. (4) The amount of substrate might remain the same but undergo some chemical modification making it insusceptible to the action of lysozyme.

Preliminary work had shown that one difference between sensitive and resistant strains of *M. lysodeikticus* was the greater O-acetyl content of the cell wall of the resistant strain (Brumfitt, Wardlaw and Park, 1958). To see whether this acetylation of hydroxyl groups is the sole change which determines the development of resistance of this organism, detailed cell wall analysis has been carried out on sensitive and resistant strains of *M. lysodeikticus*. Similar investigations have been carried out on another highly sensitive organism, *Bacillus megatherium*, and a partially sensitive organism *Streptococcus faecalis*. The mechanism of natural resistance by the *Staphylococcus aureus*, which has similar cell wall constituents

to those of sensitive Gram positive organisms (Cummins and Harris, 1956 ; Salton, 1958) has also been studied.

METHODS AND MATERIALS

Bacteria

The bacteria used were all taken from the standard strains kept in the laboratory.

Production of Lysozyme-resistant strains of bacteria

Single step mutation to high grade resistance did not occur with any of the lysozyme-sensitive strains of bacteria tested. Resistant strains of these bacteria were selected by serial sub-cultivation onto agar containing gradually increasing amounts of lysozyme. The cultures were incubated at 37° for 72 hr. and resistant colonies were picked off and sub-cultured on to nutrient agar containing lysozyme at the same concentration. The growth obtained was used to prepare a suspension for the inoculation of a further nutrient agar plate containing lysozyme at a higher concentration.

In this way strains of *M. lysodeikticus* were obtained capable of growth in the presence of 10 μg./ml. (strain R_3), 100 μg./ml. (strain R_2) and 4,000 μg./ml. (strain R_1) lysozyme. The parent strain (S) failed to grow in the presence of 0·1 μg. per ml. A strain of *B. megatherium* resistant to 5,000 μg./ml. lysozyme was selected whereas the parent strain was sensitive to 10 μg. per ml. and a strain of *Streptococcus faecalis* resistant to 10,000 μg. per ml. lysozyme was selected from a parent strain sensitive to 500μ g. per ml. lysozyme.

Strains were preserved by freeze drying, an ampoule being opened when required for a particular experiment.

Marking the organism

The initial experiments were carried out with *M. lysodeikticus*. Since this is a coccus commonly found in the atmosphere, it was necessary to avoid the objection that the resistant organisms which appeared on the lysozyme containing media might be the result of aerial contamination. Therefore, before making the *M. lysodeikticus* resistant to lysozyme, it was first made resistant to 100 μg./ml. streptomycin. In addition an antiserum was prepared against *M. lysodeikticus* which was found to agglutinate sensitive and resistant strains to the same titre but which showed no cross reactions with other air cocci.

Preparation of Cell Walls

The bacteria were grown by surface culture on nutrient agar, harvested, washed three times in distilled water and then disrupted by treatment in the Mullard sonic generator. Samples were removed at intervals and stained by Gram's method. When the majority of intact organisms had disappeared the material was centrifuged at 2,000 r.p.m. for 15 min. to remove intact bacteria. The cell walls were then deposited by centrifugation at 10,000 r.p.m., washed twice in distilled water and freeze-dried. Each batch of cell walls was checked by electron microscopy and the absence of nucleic acid proved spectrophotometrically.

Analysis of Cell Walls

Qualitative analysis

The cell walls were first hydrolysed according to the method of Cummins and Harris (1956). Two dimensional chromatograms (Whatman No. 4) were run using phenol-ammonia followed by butanol-acetic acid-water (4:1:5). Amino sugars were identified by Smith's (1958) modification of the Elson-Morgan method and sugars by the use of aniline hydrogen phthalate (Partridge, 1949) and the silver nitrate method of Trevelyan *et al.* (1950). Amino acids were detected by spraying with 1 per cent ninhydrin in butanol.

Quantitative analysis

Sugars and amino sugars.—10 mg. cell walls were hydrolysed in 0·4 ml. 2N. HCl at 120°. Passage through columns of Dowex 50(H^+) was used to remove amino acids and amino sugars. The eluate was examined for total carbohydrate by the anthrone method (Seiffer

et al., 1950), for reducing sugar (Folin and Wu, 1920) and glucose (Huggett and Nixon, 1957). The remainder of the eluate was dried in vacuo and used for chromatography.

Muramic acid and glucosamine were eluted from the Dowex 50 (H^+) and estimated by the method of Park (1958).

Amino acids were estimated by elution of ninhydrin stained spots (Kay, Harris and Entenman, 1956).

Inorganic phosphorus was estimated by the method of Allen (1940).

O-acetyl.—O-acyl estimations were made by an adaptation of the method of Hestrin (1949). Paper chromatography (Burness and King, 1958; Thompson, 1951) was used to identify the acyl groups as acetyl using both free acids and hydroxamates as standards.

Ribitol phosphate derivatives (Teichoic acids) were identified and estimated by the methods of Armstrong *et al.* (1958).

Lysozyme

The majority of tests were carried out with crystalline egg-white lysozyme (Armour) but tests were also carried out using " lysozyme-like " substances: *Bacillus subtilis* " lysozyme " (Richmond, 1957) and human leucocyte " lysozyme ".

(Throughout this paper " lysozyme " when used without qualification refers to crystalline egg white lysozyme).

Determination of Sensitivity to Lysozyme

Reduction in turbidity

Changes in optical density of a bacterial suspension were followed in the Spekker absorptiometer using grey filters. These lysis tests were carried out in 0·1 M phosphate buffer pH 7 at 37°.

Liberation of reducing substances

Serial estimations were made by Hanes, (1929) modification of the method of Hagadorn and Jensen.

Release of hexosamine end groups

This is the most specific method of following the action of lysozyme. Release of N-acetyl hexosamine end groups was determined by the method of Reissig, Strominger and Leloir (1955) and glucosamine estimations were made by the method of Rondle and Morgan (1955).

Electronmicroscopy of sections of bacteria

The morphological changes which result from lysozyme action were studied by electronmicroscopy. By setting up a number of identical tests it was possible to study the changes produced by lysozyme at varying intervals of time. To stop the action of the enzyme a few drops of 2 per cent osmic acid were added and the preparation rapidly cooled to 0°. After concentration by centrifugation, the bacteria were fixed, dehydrated and embedded in butyl methacrylate. Sections were cut with the ultramicrotome.

RESULTS

Development of Resistance by M. Lysodeikticus

Demonstration of the resistance

It was found that suspensions of *M. lysodeikticus* capable of growth in the presence of high concentrations of lysozyme were slowly lysed when a suspension of the organism in 0·1 M phosphate buffer was exposed to 1 μg. per ml. lysozyme. The rate of lysis depended upon the extent of the resistance of the organism (Fig. 1). The slow lysis of strain R_1 which is capable of growth in the presence of 4,000 μg. lysozyme per ml. requires comment.

When a washed suspension of *M. lysodeikticus* is suspended in 0·1 M phosphate

buffer at 37° no growth is possible and therefore cell wall synthesis ceases. Consequently the gradual destruction of cell wall material which follows exposure to 1 μg./ml. lysozyme causes a decrease in the opacity of the suspension. If the bacteria were actively growing, new cell wall material would be incorporated constantly and so compensate for the damage caused by the lysozyme.

The effect of lysozyme on sensitive and resistant strains of *M. lysodeikticus* was studied by electronmicroscopy. Before exposure to lysozyme sections of

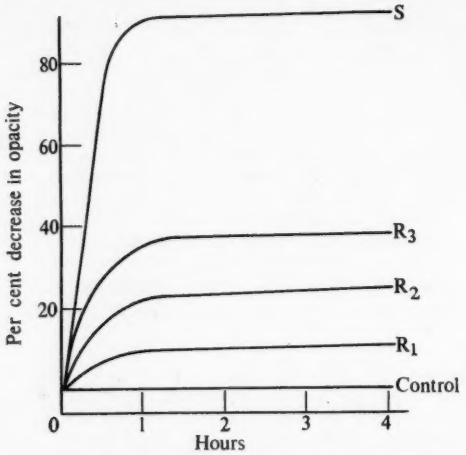


FIG. 1.—Lysis by lysozyme of sensitive and resistant strains of intact cells determined by reduction in optical density.

S : *M. lysodeikticus* incapable of growth in the presenceof 0·1 μg. per ml. lysozyme.
R_1 : Mutant capable of growth in the presence of 4,000 μg. per ml. lysozyme.
R_2 : Mutant capable of growth in the presence of 100 μg. per ml. lysozyme.
R_3 : Mutant capable of growth in the presence of 10 μg. per ml. lysozyme.

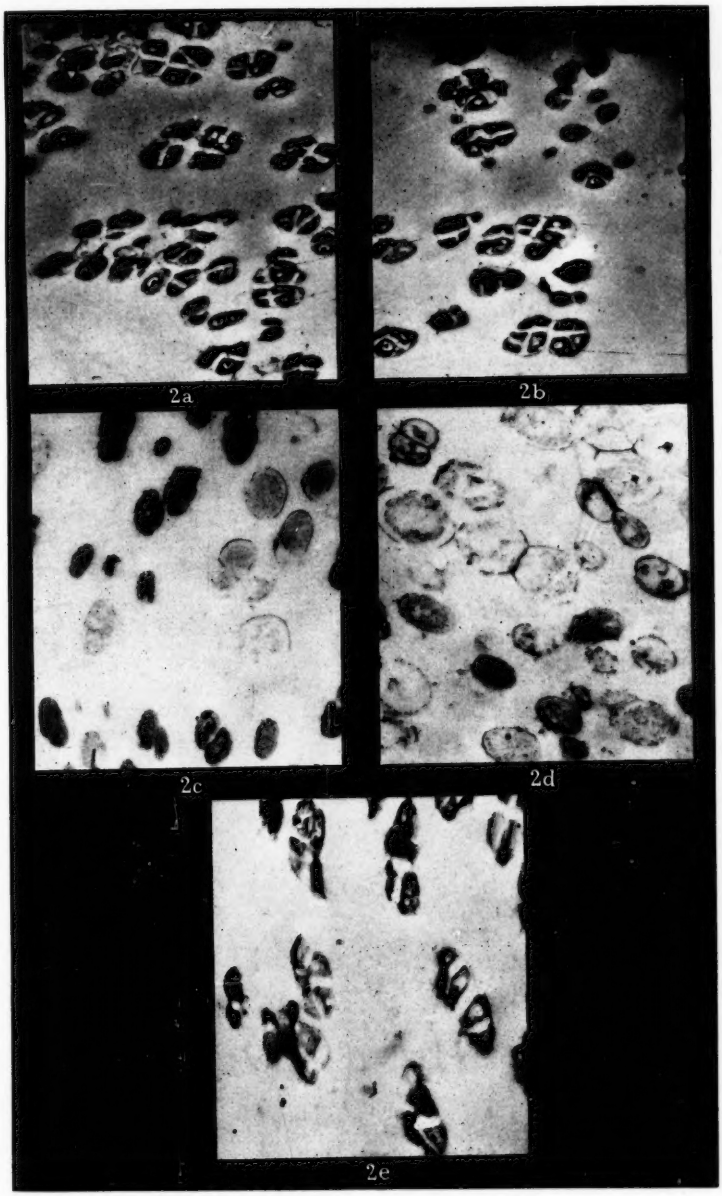
strain S (Fig. 2*a*) and strain R_1 (Fig. 2*b*) are indistinguishable. On the addition of lysozyme, strain S is rapidly destroyed. After only 30 sec., cell wall destruction and bacterial lysis can be seen (Fig. 2*c*) and after 5 min. it is extensive—few cells remaining intact (Fig. 2*d*). In contrast after 4 hr. the majority of the resistant organisms are intact, although there is some reduction in cell wall density (Fig. 2*e*).

When cell walls prepared from S and R_1 were exposed to lysozyme, lysis of the sensitive strain was more rapid and more complete (Fig. 3). Using more concentrated cell wall suspensions and 10 μg. per ml. lysozyme estimations were

EXPLANATION OF PLATE.

FIG. 2.—Electronmicrographs of sections of *M. lysodeikticus* to show the effect of treatment with 1 μg. lysozyme per ml.

(*a*) Strain S before treatment. (*b*) Strain R_1 before treatment. (*c*) Strain S 30 sec. after exposure to lysozyme. (*d*) Strain S 5 min. after exposure to lysozyme. (*e*) Strain R_1 4 hr. after exposure to lysozyme.


2a
2b
2c
2d
2e

made of release of reducing substances (Fig. 4) and of hexosamine end groups (Fig. 5) by the action of lysozyme and once again clear cut differences between sensitive and resistant cell walls can be seen. Results similar to those found with

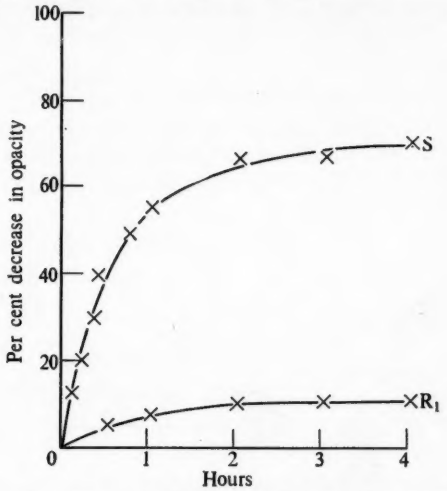


FIG. 3.—Lysis by lysozyme action of cell walls of strains S and R_1.

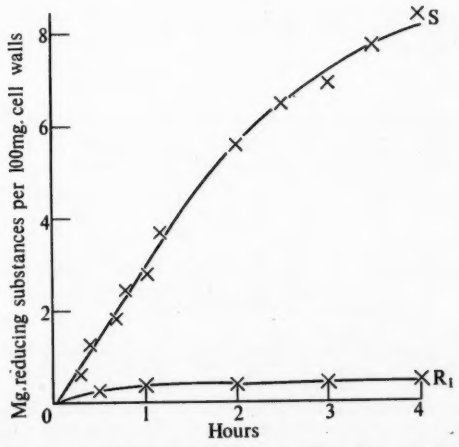


FIG. 4.—Release of reducing substances by lysozyme action from cell walls of strains S and R_1.

egg white lysozyme were obtained with *B. subtilis* "lysozyme" and leucocyte "lysozyme".

The graphs shown in Fig. 1, 3, 4 and 5 were constructed from the results obtained in a series of compatible experiments.

The nature and properties of the resistant strain

The colonial appearances and stained preparations of sensitive and resistant strains of *M. lysodeikticus* were almost identical. However, there was a considerable difference in the rate of growth between the sensitive and resistant strains.

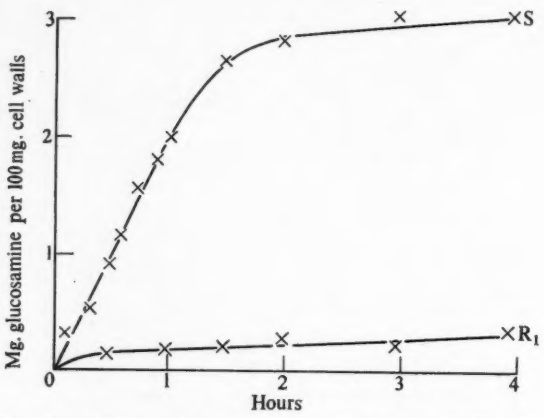


FIG. 5.—Release of hexosamine end groups by lysozyme action (estimated as glucosamine) from cell walls of strains S and R_1.

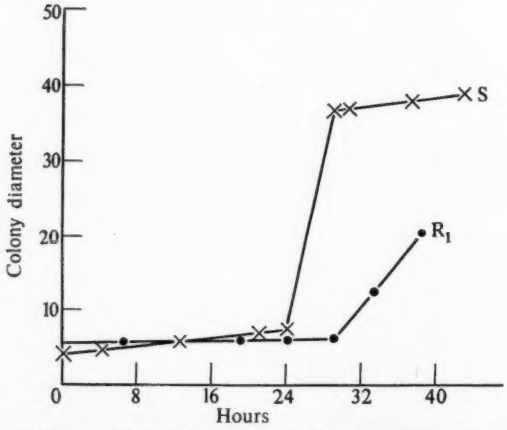


FIG. 6.—Growth curve for strains S and R_1 determined by selection of single organisms using the micromanipulator and plotting the increase in colony diameter. The units for colony diameter are arbitrary and are based on those of a standard eyepiece micrometer (magnification × 360).

The growth curve obtained by removing single cocci of the sensitive and resistant strains with the micromanipulator and seeding onto nutrient agar followed by measurement of mean colony diameter (Hughes, 1955) is shown in Fig. 6.

Differences similar to those shown in Fig. 6 were found when the increase in

viable count was plotted against time. The rate of growth varied inversely with the level of lysozyme resistance.

Experiments using the replica plating technique of Lederberg and Lederberg (1952) and the fluctuation test (Luria and Delbrück, 1943; Demerec, 1948) provided evidence that the lysozyme resistance is due to selection of a mutant.

Analysis of the cell walls of the fully sensitive strain and highly resistant strain (R_1)

Chromatography of the cell walls of the two strains, after hydrolysis showed an identical pattern for amino acids, amino sugars and hexoses. A diagram of a typical chromatogram sprayed with ninhydrin is shown in Fig. 7.

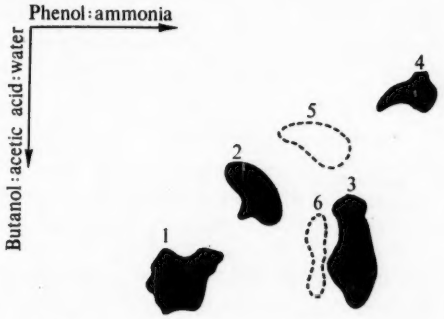


FIG. 7.—Typical chromatogram (sprayed with ninhydrin) of cell walls of *M. lysodeikticus* hydrolysed with 2N.HCl at 120°C. for 2 hr. (an identical pattern given by sensitive and resistant strains.)

The results of quantitative analysis of the two strains are shown in Table I. Figures are expressed as percentages of the total weight. Table I shows clearly that the only significant difference between sensitive and resistant strains is in the O-acetyl content of the cell wall.

TABLE I.—*Composition of the Cell Walls of Sensitive and Resistant Strains of* M. lysodeikticus

Component	Percentage by weight	
	Sensitive Strain	Resistant Strain (R_1)
Glucosamine	8·7	8·4
Muramic acid	12·2	12·6
Glucose	3·2	3·6
Galactose	1·8	1·5
Alanine	21·0	19·0
Glycine	16·0	17·0
Lysine	12·0	11·0
Glutamic acid	14·0	14·0
Inorganic phosphorus	0·17	0·19
Ribitol phosphate derivatives	0	0
O-acetyl	0	2·9

Effect of acetylation and deacetylation

O-acetyl is alkali labile and, therefore, by treatment with 0·01 N NaOH it was possible to remove the acetyl group from the cell wall of the resistant strains. After deacetylation the previously lysozyme-resistant cell walls became sensitive to the action of lysozyme. If sensitive cell walls were O-acetylated by treatment with acetic anhydride they became resistant to lysozyme, if deacetylated once again lysozyme-sensitivity was restored (Table II).

TABLE II.—*Comparison of O-acetyl Content and lysozyme Sensitivity of Cell Walls of Sensitive (S) and Resistant (R_1) Strains of* M. lysodeikticus *and the Effect of Acetylation of S and Deacetylation of R_1*

Strain	Treatment	O-Acetyl content of cell wall (μM/gm.)	Lysozyme Sensitivity: Percentage reduction in optical density (After 90 min. at 37°.)	Lysozyme Sensitivity: Glucosamine release mg. per cent cell walls (After 90 min. at 37°.)
S	None	0	72	3·1
S	Acetylation	295	28	0·7
R_1	None	490	10	0·3
R_1	Deacetylation	6	64	2·3

By using an alkaline buffer at pH 11·4 it was possible to partially deacetylate intact cells of strain R_1 without loss of viability. After treatment at pH 11·4 lysozyme caused a reduction in optical density of 75 per cent after 2 hr. whereas a fall of only 10 per cent took place after exposure to a buffer at pH 7. Subculture of cells rendered sensitive to lysozyme by deacetylation produced colonies, the cells of which were once more lysozyme resistant.

Production of a lysozyme inhibitor

Experiments in which known concentrations of lysozyme were incubated with the resistant strain of *M. lysodeikticus* R_1 failed to show any inactivation of the enzyme.

Cell Wall Changes Occurring During the Development of Lysozyme Resistance by Other Bacteria

B. megatherium.—The findings were similar to those with *M. lysodeikticus*. Analysis of lysozyme-sensitive and resistant cell walls showed no significant difference in any constituent other than the O-acetyl content (sensitive—0·1 per cent, resistant—2·3 per cent). The resistant strain could be made sensitive by deacetylation and the sensitive strain resistant by O-acetylation.

Strep. faecalis.—Analysis of the cell walls of a lysozyme sensitive strain of streptococcus faecalis showed an O-acetyl content of 0·5 per cent. However, deacetylation failed to increase the sensitivity to lysozyme. On the other hand, when strains of the same organism which had been made highly resistant to lysozyme were tested, the O-acetyl content was found to be 2·4 per cent. Deacetylation restored the cell walls to their original level of lysozyme sensitivity.

Natural Resistance to Staph. Aureus

The O-acetyl content of the cell walls was 2·0 per cent. Following deacetylation the organism remained totally resistant to the action of lysozyme.

The cell wall contained 38 per cent ribitol phosphate derivatives.

Pathogenicity of Lysozyme Resistant Strains of M. Lysodeikticus *and* Strep. Faecalis

In phagocytosis experiments it was found that in the presence of serum both sensitive and resistant strains were ingested by polymorphs. Within the polypolymorphs the sensitive strains were lysed and destroyed, whereas the resistant strains were not lysed and remained viable. However, in mice no difference could be shown between the pathogenicity of sensitive and resistant strains either by intraperitoneal challenge or by the technique of intramuscular injection into the thigh described by Selbie and Simon (1952).

DISCUSSION

The results show that lysozyme-resistant variants of *M. lysodeikticus* and *B. megatherium* differ from the parent strain in their ability to manufacture cell wall with acetylated hydroxyl groups. Feiner, Meyer and Steinberg (1946) suggested that the lysozyme-resistance of *M. lysodeikticus* might be due to a decrease in lysozyme substrate but in the present investigation no change, other than in O-acetyl content, was found in the cell wall components of the resistant strains.

When the O-acetyl content of the fully resistant strain of *M. lysodeikticus* (R_1) is compared on a molar basis to the disaccharide, N-acetyl muramic acid and N-acetyl glucosamine, it is found to bear a 1:1 ratio. It has previously been proposed (Brumfitt, Wardlaw and Park, 1958) that lysozyme acts by splitting a bond (β1-4 linkage) between N-acetyl glucosamine and N-acetyl muramic acid. The observation that in the resistant strain there is approximately one O-acetyl group per disaccharide unit suggests that acetylation of a surface hydroxyl group prevents lysozyme acting upon the sensitive bond. One explanation for this finding is that, in order to act upon its substrate, the enzyme requires a three point attachment and by acetylation of hydroxyl groups such an attachment is prevented. Similar inhibition of enzyme action following substitution of substrate hydroxyl groups is found with the glycosidases (Pigman, 1957).

Deacetylation of lysozyme-resistant strains of *M. lysodeikticus* or *B. megatherium* restores the sensitivity and this change can again be reversed by reacetylation. Therefore, sensitivity or resistance to lysozyme can be determined by simple chemical treatment. The genetic change which enables organisms to acetylate cell wall hydroxyl groups and so become lysozyme resistant slows the growth rate of the organism (Fig. 6). As a result, sensitive mutants overgrow the resistant organisms and this favours back-mutation, although complete reversion does not occur.

O-acetylation is apparently not the means by which pathogenic bacteria are naturally resistant to lysozyme. Experiments with the *Staph. aureus* which contains an N-acetyl glucosamine-N-acetyl muramic acid polymer showed that although O-acetyl is a constituent of the cell wall, deacetylation failed to make

the organism lysozyme-sensitive. One reason for this finding would be that lysozyme sensitive bonds are absent from the cell wall, but the observations of Kern *et al.* (1951) and Mitchell and Moyle (1957) suggest that this is not true for all strains of staphylococci.

It is interesting to speculate on the role of ribitol phosphate derivatives (teichoic acids), described by Armstrong *et al.* (1958) in the determination of natural resistance of bacteria to lysozyme. The cell wall of *M. lysodeikticus* was found to contain no teichoic acid but this substance accounted for 38 per cent of the *Staph. aureus* cell walls used in the present investigation. Teichoic acids are insusceptible to lysozyme but it is possible that their presence in the cell wall may interfere with the action of lysozyme upon its substrate. A lysozyme sensitive strain of *B. subtilis* had a cell wall teichoic acid content of 54 per cent, but the teichoic acid from *B. subtilis* differs from that of *S. aureus* in containing glucose instead of N-acetyl glucosamine side chains (Armstrong *et al.* (1958). Clearly the rôle of cell wall teichoic acids in lysozyme resistance must await further investigation.

SUMMARY

Changes occurring in the cell wall of some gram positive organisms following the development of resistance to lysozyme have been investigated. Highly resistant variants of *M. lysodeikticus*, *B. megatherium* and *Strep. faecalis* were obtained by serial subculture on nutrient agar containing increasing amounts of egg-white lysozyme. Detailed chemical analysis of cell walls prepared from sensitive and resistant strains of *M. lysodeikticus* and *B. megatherium* showed that acetylation of hydroxyl groups of the substrate polymer was apparently the sole change which determined resistance. Therefore the genetic modification of lysozyme resistant strains of *M. lysodeikticus* and *B. megatherium* is in the ability to acetylate cell wall hydroxyl groups.

The cell wall polymer which forms the lysozyme substrate is composed of two amino sugars, N-acetyl glucosamine and N-acetyl muramic acid. In the most resistant strain of *M. lysodeikticus* there was a 1:1:1 molar relationship between N-acetyl glucosamine, N-acetyl muramic acid and O-acetyl.

Acetylation of hydroxyl groups was found to play no part in the natural resistance of *Staph. aureus* to lysozyme.

Conventional pathogenicity tests were used to compare resistant and sensitive strains of *M. lysodeikticus* and *Strep. faecalis*. Lysozyme-resistance did not enhance the virulence of these two organisms.

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POLIOVIRUS D AND C ANTIGENS: THEIR DIFFERENTIATION AND MEASUREMENT BY PRECIPITATION IN AGAR

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POLIOVIRUS preparations of any given strain may contain several particulate forms differing in size, density, chemical composition, infectivity and antigenic character (Schwerdt and Schaffer, 1956; Roizman, Mayer and Rapp, 1958; Polson, Ehrenberg and Cramer, 1958*a*, 1958*b*; for other references see Schaffer and Schwerdt, 1959). Immunologically, such particles are of 2 kinds, designated D or C according to their predominant antigens. These antigens are named after the two physical fractions of purified poliovirus with which they are characteristically associated—fractions SDG^D and SDG^C, separated by sedimentation of partially purified, concentrated virus in the sucrose density gradient (Schwerdt and Schaffer, 1956).

When tested with sera from monkeys immunized with one type of poliovirus, the D and C particles give type-specific reactions, both by complement fixation (Mayer *et al.*, 1957) and by precipitation in agar (Le Bouvier, Schwerdt and Schaffer, 1957).

The D antigenic structure is characteristic of virus particles that are infective, appear well defined and uniform in electron micrographs and contain some 25–30 per cent of ribonucleic acid (Schwerdt, 1957). Possesion of the D structure, however, does not of itself connote infectivity, for the majority of D-antigenic particles are non-infective for the animal hosts or cultured cells available. In addition, Roizman, Mayer and Rapp (1958) have described a third group of D-antigenic particles, sedimenting with the C particles and, like them, non-infective. With respect to their type-specific reactivity these 3 kinds of D particles are collectively referred to as "D antigen".

The C antigenic configuration is associated with particles which are less dense than the majority of the D particles, appear structurally impaired by electron microscopy, and are largely or wholly deficient in ribonucleic acid. The type-specific component of C particles, and that of the antigenically indistinguishable particles which may be present in the more slowly sedimenting fraction SDG^B, together constitute "C antigen".

A further distinction between D and C antigens lies in the capacity to evoke neutralizing antibodies, which either depends on, or runs parallel with, an unmodified D configuration (Powell and Culbertson, 1955; Le Bouvier, 1955; Hummeler and Hamparian, 1958).

Many poliovirus preparations, whether crude infected tissue-culture fluids or partially purified concentrates, contain not only D antigen but also varying amounts of C (Roizman, Höpken and Mayer, 1958; Hummeler and Hamparian,

1958). The proportion of " spontaneous " C antigen is probably related to the character of the host-cell, the conditions of virus growth and the subsequent handling of the material. It is not known whether or to what extent C antigen may be synthesized in infected cells, either as a precursor of D or as a separate end-product; but it is evident, from the studies of Mayer and his colleagues using complement fixation, that C, or a closely related, antigen is produced or revealed and the D correspondingly diminished, when preparations of D-antigenic particles are heated or irradiated with ultraviolet light. As there are, so far, no demonstrable immunological differences between such modified preparations and the C antigen of purified fraction SDG^C, this " heated-D antigen " has been provisionally accepted as yet another form of " C antigen "; and the modification of poliovirus suspensions by heat, ultraviolet light and other agents is here designated the D→C change. Some aspects of this change will be considered in a subsequent paper.

The present paper deals with the means of distinguishing and measuring the two kinds of antigens by their precipitation reactions in agar with antisera from immunized animals. The relationship and behaviour of the D and C antigens of poliovirus are presumably important factors in vaccine production and human serology. Purified poliovirus fractions have not yet replaced crude preparations in these 2 fields; it has therefore seemed reasonable to use unpurified concentrates of tissue-culture virus for the greater part of the work here described. The method of agar-diffusion is well suited to the study of such crude materials. Though rather insensitive, compared, for example, with complement fixation, it has the advantage of allowing the different components in a mixture to be distinguished and estimated without first having to be separated by fractionation or absorption.

MATERIALS AND METHODS

Virus.—Most of the work has been done with concentrates of a single strain of poliovirus, the Type 1 Brunhilde strain, grown in a line of aneuploid cells (HuLi) obtained from cultures of human embryonic liver (Westwood, Macpherson and Titmuss, 1957; Westwood and Titmuss, 1957). Type 2 and Type 3 polioviruses have been represented by the Y-SK and Leon strains, respectively.

Virus-infected culture liquid, produced as described below, was clarified by spinning at 1500 r.p.m. for 15 min. The supernatant was spun for 1 hr. at 39,000 r.p.m. (average RCF = 100,000 g) in the No. 40 rotor of the Spinco Model L ultracentrifuge. The resulting pellets were re-suspended in a volume of diluent equal to 1/50 of the original volume of infected culture fluid. The diluent consisted of 0·14 M sodium chloride solution containing 0·01 M phosphate, pH 7·3. Such virus preparations, referred to as " 50-fold concentrates ", were kept at 4° for up to 3 weeks or stored at −23° for longer periods.

In parallel with crude concentrates, some comparative tests have been done on purified poliovirus fractions kindly supplied by Dr. F. L. Schaffer, of the University of California Virus Laboratory, at Berkeley (Schwerdt and Schaffer, 1956).

Control antigen preparations have included the various medium constituents and extracts of the host cells. For the latter, confluent uninoculated cultures of HuLi cells were left at 37° without change of medium for a week. The detached and degenerating cells were then ground in a ten Broeck grinder and resuspended in 1/10 volume of their old medium.

Sera.—Rabbit and monkey antisera have been used. Rabbit sera were heated at 56° for 1 hr., unless otherwise stated. Monkey sera were used unheated. Small aliquots of serum were stored at 4°, larger quantities at −23°.

Rabbit antisera employed were those in current use in this laboratory (Smith, Sheffield, Lee and Churcher, 1956). The immunizing antigens comprised concentrates of Type 1 Brunhilde, Type 2 Y-SK and Type 3 Leon viruses grown in HeLa cells, or in cell lines (HuLi, ERK) derived from cultures of human embryonic liver or embryo-rabbit kidney (Westwood

et al., 1957). One particular pool of Brunhilde rabbit antiserum was adopted as a " standard " reactant for all comparative tests of Brunhilde virus concentrates : unless otherwise stated, it was used at a dilution of 1/8. Antisera were also obtained against Brunhilde virus heated at 60° for various lengths of time.

Monkey antisera were kindly provided by Drs. F. O. MacCallum and G. P. B. Boissard of the Virus Reference Laboratory, Colindale, and by Dr. E. M. Opton of the Section of Epidemiology and Preventive Medicine, Yale University School of Medicine. The rhesus monkeys had had 2 or more intramuscular injections of unheated unconcentrated liquids from poliovirus-infected rhesus-kidney cultures, with or without emulsification in Bayol-Arlacel adjuvant. The virus strains used were Brunhilde (Type 1), Y-SK and MEF-1 (Type 2), and Leon and Saukett (Type 3).

Cell cultures and production of virus.—The HuLi cell line was used both for production and titration of virus, and for assay of neutralizing antibodies in rabbit sera. The method of virus propagation was that described by Sheffield and Churcher (1957), with a few modifications.

Confluent cell-sheets were removed from the glass with 0·01 per cent versene in phosphate-buffered saline, made up without calcium and magnesium salts (Dulbecco and Vogt, 1954). After dispersal by pipetting, the cells were sedimented at 700 r.p.m. for 5 min. and suspended in medium at a concentration of 100,000–200,000 cells/ml. The medium used both for growth of cells and for maintenance after infection consisted of Earle's balanced salt solution, containing 0·25 per cent lactalbumin hydrolysate, 5 per cent tryptic digest meat broth, and 10 per cent horse serum free from poliovirus inhibitors. Penicillin, 100 units/ml., and streptomycin, 100 μg./ml., were added. The pH was adjusted with 2·78 per cent sodium carbonate solution : for fresh cultures, 2 ml. were added to 100 ml. of medium, and the mixture saturated with CO_2 ; for established cultures, 3 or 4 ml. of sodium carbonate solution were added, and the medium used without gassing.

Cultures were made in 8 oz. Pyrex babies' feeding bottles, using 10 ml. of cell suspension per bottle. The bottles were kept stationary at 37° for 3 days, after which the medium was changed, and incubation continued for a further 1 or 2 days. When the glass was thickly covered with cells (5–10 million per bottle), the medium was renewed, each culture inoculated with about 1 million 50 per cent infective doses (TCD_{50}) of virus and the bottles then rolled. Cultures infected with Types 1 and 3 viruses were left for 2–3 days, those infected with Type 2 virus for 3–4 days before harvesting.

Virus titration.—Dilutions of virus fluids under test were made in Earle's saline containing 0·25 per cent lactalbumin hydrolysate and 5 per cent tryptic meat broth, adjusted with 2·78 per cent sodium carbonate to pH 7·0. Infectivity titrations were done in tube cultures made from suspensions of HuLi cells prepared as described above, 0·2 ml. volumes of serial 10-fold dilutions of virus being inoculated into each of five tubes. Final readings were made on the 5th day and the 50 per cent end-point calculated by Kärber's method (1931).

Titration of neutralizing antibody.—Neutralizing antibodies were titrated in 3-day-old tube cultures of HuLi cells. Five-fold dilutions of serum were mixed with equal volumes (0·8 ml.) of virus containing an estimated 100 TCD_{50} per 0·1 ml. Mixtures were incubated for 1½ hr. at 37°, and 0·2 ml. inoculated into each of 2 tubes which were then rolled at 37°. The end-point was taken as the initial dilution of serum which reduced the virus infectivity to 1 TCD_{50}. Readings were made at 2–3 and at 5–6 days, to observe both the transitory and the more durable neutralizing activity of the serum (Sabin, 1959).

EXPLANATION OF PLATE.

FIG. 1.—D and C antigens of Type 1 poliovirus.

1 = Brunhilde virus 50× concentrate, unheated.

1′ = the same concentrate, heated at 60° for 20 min.

i, ii, iii } Brunhilde, Y-SK and Leon monkey antisera, diluted ½.

FIG. 2.—Absence of reciprocal antibody cross-absorption by Type 1 poliovirus D and C antigens.

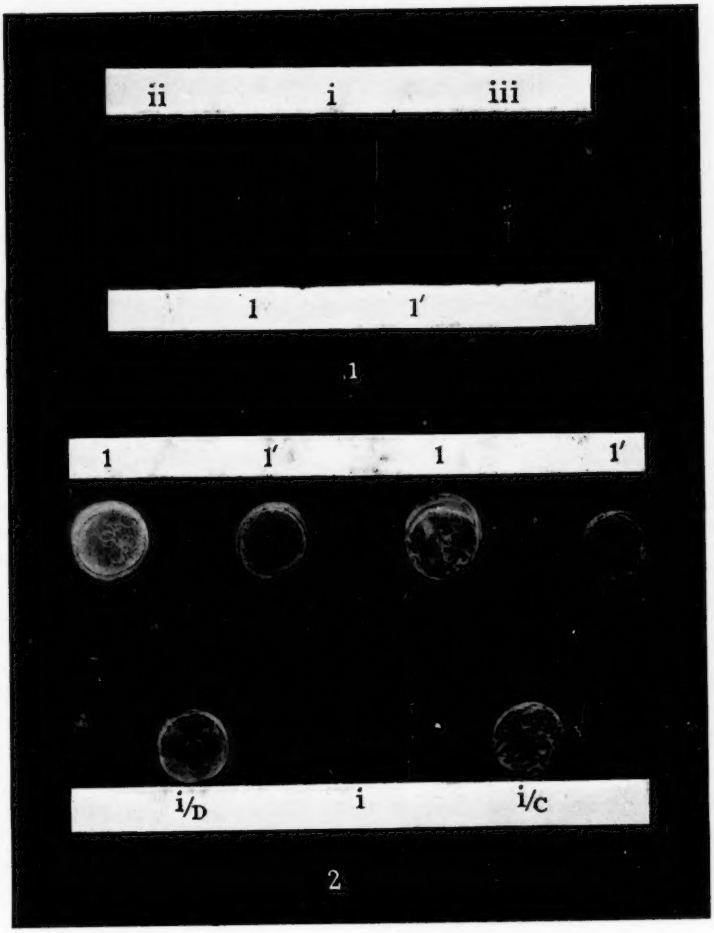
1 = Brunhilde virus 50× concentrate, unheated.

1′ = the same virus concentrate, heated at 60° for 20 min.

i = unabsorbed Brunhilde antiserum, diluted 1/6.

i/D = antiserum absorbed with 1

i/C = antiserum absorbed with 1′



Le Bouvier.

Agar-diffusion tests.—The method of two-dimensional double diffusion was used throughout (Le Bouvier, 1957), with the following modifications. Larger plates, 7 cm. in diameter, were used, and the volumes of Difco-" Bacto " agar were increased to 5 ml. and 14 ml. for the foundation and reaction layers, respectively. The agar, made up in 0·01M phosphate-buffered saline at pH 7·3, often contained a deposit due to calcium phosphate, but versene was found to clear the agar effectively and was therefore added in the later tests to a final concentration of 0·1 per cent. This concentration of versene did not inhibit the specific precipitation of poliovirus reactants but it made the gel less firm, and so the percentage of agar in the reaction layer was increased from 0·5–0·7 per cent. The pH was readjusted to 7·3 with 1 N NaOH, a small amount of phenol red being added to the agar as indicator.

All cups were cut 3·5 mm. in diameter and the spacing between virus and serum cups was usually 7·5 mm., but occasionally less. With this arrangement, specific precipitates were visible after 2 days' incubation at 37°. The position of their leading edges was determined on the 3rd day by measurements made on photographs. For photography, plates were flooded with 5 ml. of a 0·03 per cent solution of Orange G (Wilson and Pringle, 1955).

The means of distinguishing D and C antigenic components, and of assaying the corresponding antibodies, are described below.

RESULTS

D and C precipitation reactions

Many crude concentrates of poliovirus give two bands of precipitate, " major " and " minor ", when allowed to diffuse in agar towards homotypic monkey or rabbit antisera. The relative concentrations of the two, type-specific, antigenic components may differ greatly from one preparation to another, as judged by the position and density of their precipitates (Le Bouvier, 1957). In some concentrates a " minor-band " component is undetectable.

Purified sucrose-density-gradient fractions, SDG^D and SDG^C, of Type 1 (Mahoney) and Type 3 (Saukett) polioviruses were tested alongside crude 50-fold concentrates of homotypic viruses of the Brunhilde and Leon strains respectively. With each virus type, there was clear and complete fusion between the precipitate given by the SDG^D fraction and the major band of the crude preparation, and between that given by the SDG^C fraction and the minor band. The " D " and " C " bands, however, remained distinct, intersecting without any sign of fusion.

When the purified SDG^D fractions and the crude concentrates were heated at 60° for 20 min. their respective D and major bands were no longer seen. Instead, each preparation produced a single band of precipitate which fused with those given by fraction SDG^C and by the spontaneously occurring minor component of unheated homotypic concentrates. Heating fraction SDG^C at 60° for 20 min. did not change the character of the band. No differences in reactivity have been found between the four kinds of C-antigenic material mentioned: SDG^C, heated SDG^D, the minor component of unheated crude virus concentrates, and such concentrates heated at 60° for 20 min. Moreover, no serum yet tested, that has reacted with the minor component, has failed to give a similar precipitate with heated virus of the same type, and absorption of such serum with heated virus abolishes its reactivity with both heated virus and the minor component of unheated concentrates. It is therefore concluded that the C precipitation given by the 4 different kinds of antigenic material is due to a single antigen or to antigens so closely related as to be indistinguishable by the tests so far applied. The same conclusion was reached in respect of the poliovirus C complement-fixing antigens by Roizman and his colleagues (Roizman, Mayer and Rapp, 1958; Roizman, Mayer and Roane, 1959).

In Fig. 1, an unheated Brunhilde virus concentrate is seen to form two bands, D and C, with a Type 1 monkey antiserum, but none with the heterotypic antisera. The same concentrate after heating at 60° for 20 min. produces a single band which fuses with the C, or minor, band of the unheated virus, but which is denser and further removed from the virus cup. This indicates that while heating has suppressed or destroyed the D precipitating antigen, it has led to an apparent increase in the concentration of the C.

Absorption experiments

Various, and at first sight conflicting, results have been obtained in cross-absorption tests with monkey and rabbit antisera, using unheated and heated poliovirus concentrates of the same type. Sera from different individual animals have reacted differently with identical virus absorbents: where one serum has indicated that the D and C antigens, and their antibodies, are distinct, another has shown cross-absorption of varying degree between the 2 kinds of preparation. The reasons for these apparent anomalies are not yet understood and are under investigation. Fig. 2 shows the results of a test with Type 1 (Brunhilde) reactants. One part of a monkey antiserum, diluted $\frac{1}{2}$, was mixed with 2 parts of virus concentrate, either unheated or heated at 60° for 20 min. The heated concentrate reduced the anti-C precipitins to undetectable levels without absorbing the anti-D. The unheated concentrate absorbed out the anti-D antibodies; it also caused a relatively small reduction of the anti-C, probably because it contained some "spontaneous" C antigen. The same concentrates were used both as absorbents and as reactants in the test.

The same Type 1 antiserum, absorbed with either unheated or heated virus, was used in further absorption tests with 2 suspensions containing both D and C homotypic antigens: the first was an unheated virus concentrate possessing "spontaneous" C antigen as well as D; the second consisted of the same concentrate heated at 50° for $1\frac{1}{2}$ min., a degree of heat which caused a partial conversion from D reactivity to C. The "anti-D serum", absorbed with heated virus, reduced the level of D antigen in each preparation while producing no significant change in the C. Conversely, the "anti-C serum," absorbed with unheated virus, removed C antigen from the two suspensions without appreciably affecting the content of D.

Absorption of the Type 1 monkey antiserum with virus concentrates of Types 2 and 3, either unheated or heated at 60° for 20 min., caused no detectable reduction in the concentration of diffusible Type 1 precipitins, whether anti-D or anti-C.

These results are analogous to those of Hummeler and Hamparian (1958), who found that unheated and heated preparations of fluorocarbon-purified HeLa-grown poliovirus did not absorb the heterologous complement-fixing antibodies from the sera either of young children with poliovirus infections, or of monotypically immunized chimpanzees, monkeys and guinea-pigs. They showed that in such sera the complement-fixing antibodies against both unheated and heated virus were type-specific. Another of their findings was confirmed: full neutralizing titre, at both the 2nd and 5th day reading, was retained by a hyperimmune antiserum after absorption with heated, homotypic virus. A Brunhilde monkey serum, diluted $\frac{1}{2}$, was mixed in the proportion of 1 : 4 with 50-fold Brunhilde virus concentrate which had been heated at 60° for 20 min. The mixture was held at 37° for 2 hr., then at 4° for 18 hr., and the supernatant liquid, repre-

senting a 1/10 dilution of the serum, used without centrifugation. Whereas Type 1 anti-C precipitins were no longer detect able, both neutralizing antibodies and anti-D precipitins were present in undiminished concentration.

The immunogenic properties of D and C antigens

Brunhilde virus was grown in HuLi cells cultivated in medium containing 10 per cent of human serum instead of the usual horse serum. Before infection with virus, the cultures were washed twice with Earle's solution and given fresh medium containing 10 per cent of normal rabbit serum. Rabbits were injected intravenously with 1 ml. of the unconcentrated virus, either unheated, or heated at 60° for periods of 5, 20 or 80 min. Three weeks later, each rabbit received a second intravenous injection of the same material. Serum samples, taken before the first and second inoculations, and at intervals of 1 and 2 weeks after the second, were tested without heating for neutralizing antibodies and for anti-D and anti-C precipitins. In addition, the sera were tested for antibodies against the cellular and medium constituents present in the inocula. The only "non-specific" reactions were given by undiluted samples of serum taken after the second immunizing injection from the rabbits receiving the unheated and the 80-minute heated inocula: these produced faint precipitates, the first with the HuLi-cell extract, the second with human serum diluted 1/10 in phosphate-saline.

The method of neutralizing-antibody assay has been described above. Type 1 anti-D precipitins were titrated by testing serial two-fold dilutions of serum in plates, taking as the end-point the highest dilution giving a precipitate with unheated 50-fold Brunhilde virus concentrate. For determination of anti-C precipitins, this concentrate was heated at 60° for 20 min. The results are given in Fig. 3.

Homotypic antibody responses.—The unheated virus stimulated high levels of homotypic neutralizing antibodies, as also of anti-D and anti-C precipitins. The heated virus stimulated only about 1/1000 as much Type 1 neutralizing antibody as the corresponding unheated virus and the protective effect of this antibody was largely transitory. Similarly, the anti-D titres evoked by the 3 heated preparations were minimal. None of these unconcentrated heated preparations had any detectable D antigen, and all possessed the same amount of C antigen, judging by the precipitation reactions of comparably heated virus concentrates with the "standard" Brunhilde rabbit serum. However, each heated preparation still showed traces of infectivity. It is uncertain, therefore, whether the low levels of neutralizing and anti-D antibody should be attributed to residual, unaltered, D-antigenic virus still present in the inocula after heating, or whether they may have been evoked by the C-antigenic particles, as such. Whatever the explanation, it is clear that C antigen can, at best, be only a poor stimulator of effective homotypic neutralizing antibodies.

In contrast to the neutralizing and anti-D antibodies, almost identical levels of homotypic anti-C precipitins were produced by all 4 preparations. Even the unheated immunizing virus used in the experiments summarized in Fig. 3 was found, on concentration, to contain appreciable amounts of "spontaneous" C antigen. However, the injection of a virus concentrate which showed no detectable C component before heating likewise evoked not only neutralizing antibodies and anti-D precipitins, but also high levels of anti-C. This suggests that production,

or " uncovering ", of C antigen in inoculated D-antigenic virus suspensions may occur in the animal body.

Heterotypic antibody responses.—Sera of the 4 Brunhilde-immunized rabbits, whose Type 1 responses are shown in Fig. 3, were also tested for heterotypic neutralizing and precipitating antibodies. Here, again, the response to the unheated virus inoculum differed from those to the 3 heated inocula : after the second injection the unheated virus evoked low levels of neutralizing antibodies (titres of 5 at the 6th-day reading) against both Type 2 and Type 3 viruses, whereas

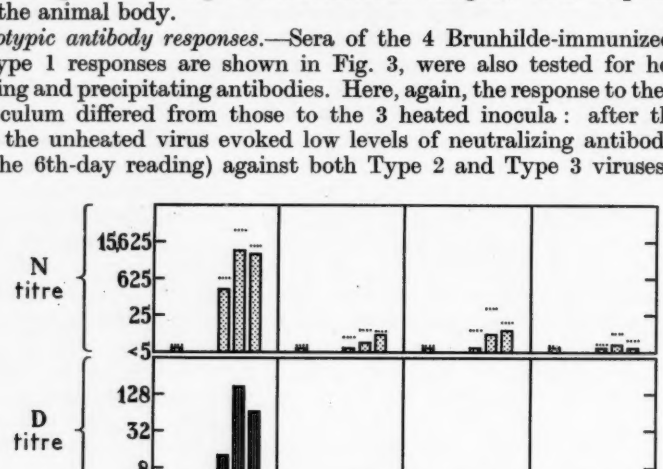


FIG. 3.—Immunization of rabbits with unheated and heated Brunhilde virus.

1 ml. of virus i.v. at day 0 and day 21 (shown by arrows) : α—unheated virus ; β, γ, δ—virus heated at 60° for 5, 20 and 80 mins., respectively. Serum collected before each injection, and 1 and 2 weeks after the second. Histograms show levels of Type 1 poliovirus antibody : N = neutralizing (6th-day reading) ; D = anti-D precipitin ; C = anti-C precipitin. Dotted lines indicate neutralizing titre at 2nd to 3rd day of test.

2 doses of the heated preparations induced no such responses. Sera of all 4 rabbits taken after the second injection were too cytotoxic to be used undiluted : the lowest dilution tested was 1/5.

The findings for heterotypic anti-D precipitins were similar to those for the neutralizing antibodies. In the undiluted serum of the rabbit immunized with unheated virus, traces of precipitins against Types 2 and 3 could be detected, whereas the other 3 rabbits showed none. In contrast, heterotypic anti-C precipitins were stimulated by both unheated and heated inocula. All 4 rabbits had minimal levels against Type 2, and those immunized with the unheated and the 5-min. heated virus also revealed some slight Type 3 reactivity. The heterotypic character of these anti-D and anti-C precipitins was inferred from the fact that their precipitation bands fused with those formed by the interaction of homotypic Type 2

and Type 3 reactants, whereas they did not fuse with control D and C precipitates of Type 1 virus.

Chessboard titration of virus concentrate, unheated and heated

Some aspects of the measurement of poliovirus precipitating reactants in agar-diffusion tests were considered in a previous study (Le Bouvier, 1957). This dealt mainly with the " major ", *i.e.* D-antigenic, precipitating component, and monkey hyperimmune sera were used as reference standards. The present investigation has been concerned not only with D, but also with C antigen and its production by various means, and rabbit antisera have been used as reference standards. In some instances, serial dilutions of one or both reactants have been tested. But in general, tests have been done with a single concentration of virus and antiserum, placed in cups of equal size (3·5 mm. diam.) 7·5 mm. apart. Antigen or antibody has been assayed by measuring on photographs the position of the leading edges of the precipitate bands after incubation of the test for 3 days at 37°. The point between the antigen and antibody cups at which the precipitate band is formed, as also its dimensions and density, are determined by the relative concentrations of the two reactants. This is shown in Fig. 4, which presents the values obtained from a chessboard titration of a Brunhilde virus concentrate, before and after heating, against the " standard " Brunhilde rabbit antiserum. This particular concentrate, before heating, contained a small quantity of " spontaneous " C antigen, which is not shown in Fig. 4 : it was equivalent to about 1/8 of the amount present after the concentrate had been heated. Each sloping line applies to a given concentration of serum and indicates the positions of the precipitate bands which it produced with the various dilutions of antigen.

The left-hand half of Fig. 4 is concerned with the D antigen of the unheated concentrate. The slope of the various lines is about the same, corresponding to an increase of $\sim 0{\cdot}07$ in the value of x_D/y for each two-fold decrease in virus concentration, x_D being the distance of the precipitate's leading edge from the serum cup, and y the total distance between the serum and virus cups.

In the right-hand half of Fig. 4 is shown a chessboard titration of the same virus concentrate after it had been heated at 60° for 20 min. The C antigen of the heated concentrate resembles the D antigen of the unheated in the slope of its lines, but differs from it in its maximum antigen titre. That of the D preparation is 16, with production of faint bands at 1/32 with certain serum dilutions, whereas the titre of the C preparation is 4, with faint precipitates at 1/8.

A chessboard titration of the virus concentrate heated at 50° for 5 min. was indistinguishable from that seen after heating at 60° for 20 min.

As shown in Fig. 4, the lowest dilution of " standard " serum does not contain enough anti-C antibody to form a visible precipitate at the edge of the virus cup with any of the antigen dilutions used. The same heated virus dilutions were re-tested with a stronger anti-C serum, from a rabbit immunized with 60°-heated virus. The range of serum dilutions was 1/4 to 1/256, as for the "standard " serum. The lines for the various serum dilutions were now shifted nearer to the abscissa, the 1/4 serum-dilution line actually meeting it. However, the maximum antigen titre was 1/8, as in the previous test.

With a given concentration of serum, one particular virus dilution produces a band of precipitate that is clearly sharper, denser and narrower than those formed by the virus dilutions on either side of it. Such narrow bands are charac-

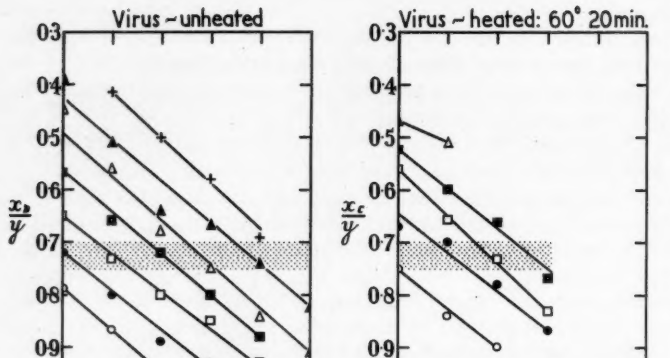


FIG. 4.—Chessboard titrations of Type 1 poliovirus D and C antigens against "standard" homotypic rabbit antiserum.

x_D, x_C = distance of leading edge of D and C precipitates, respectively, from serum cup.
y = distance between serum and virus cups.
Stippled segments denote the zones of equivalence.
Arrows mean that the leading edge of the precipitate has entered the virus cup.

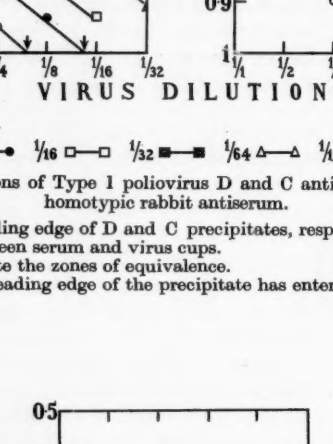


FIG. 5.—Fiducial limits of D—antigen assay.

x_D = distance of leading edge of D precipitate from serum cup.
y = distance between serum and virus cups.
Reactants: 2-fold dilutions of Brunhilde virus 50-fold concentrate (unheated); single dilution (1/8) of "standard" Brunhilde rabbit antiserum.
Each point represents mean of 4 replicate tests, $\pm$ 2 $\times$ standard error.

teristic of the aggregation of antigen and antibody in optimal proportions. Thus, by measuring the width of the various precipitates, the position of the zone of equivalence can be determined. In Fig. 4, these equivalence zones, indicated by stippled segments, have an x/y value of between 0·7 and 0·75 for both antigen-antibody systems, D and C. Most 50-fold Brunhilde virus concentrates form their D band in this zone with a 1/8 dilution of the " standard " rabbit antiserum, the one shown in Fig. 4 being typical in this respect. With the concentration of C antigen which is formed when an average 50-fold Brunhilde concentrate is heated, this 1/8 dilution of the " standard " serum is sub-optimal. Nevertheless, as it gives a reasonably clear band with most such C preparations, it has been selected as the dilution for use in all experiments on the D→C antigenic change.

Error of measurement of relative D-antigen concentration

To estimate the limits of error of antigen assays based on the position of precipitate bands, 4 tests of an identical series of virus dilutions were made on separate agar plates, using a single dilution (1/8) of the " standard " rabbit antiserum. The position of the leading edge of each D-antigenic precipitate was measured on photographs, and mean values and standard errors of x_D/y were calculated. The results are shown in Fig. 5.

DISCUSSION

By their precipitation reactions in agar, their different immunogenic properties, and the absence of reciprocal antibody cross-absorption, the type-specific D and C antigens of the Brunhilde strain of Type 1 poliovirus are seen to be distinct. When tested in the same way with monkey and rabbit antisera, other Type 1 strains, and strains of Types 2 and 3, have shown comparable differences between the two kinds of antigens. Another mark of this distinction, not considered here, is the variable pattern of anti-D and anti-C antibody responses in different individuals infected with poliovirus. The effect on the D and C antigens of treatment with various physical and chemical agents will be discussed in a subsequent paper dealing with the conversion of poliovirus suspensions from D reactivity to C, and with the relationship between particles possessing the two different antigenic configurations.

The results of the agar-diffusion experiments reported are in general agreement with the conclusions reached by other workers, particularly by Mayer *et al.* (1957), Roizman, Höpken and Mayer (1958) and Roizman, Mayer and Rapp (1958) and by Hummeler and Hamparian (1958) from their complement-fixation studies. The absorption tests suggest that in some virus concentrates, at least, the bulk of the reactive D and C precipitating antigens, besides being distinct immunologically, are also present on different particles. Other evidence obtained in this laboratory, however, indicates that this may not be true for all virus preparations (Smith, unpublished).

The existence of poliovirus antigens, common to 2 or to all 3 types, has been much debated, and further evidence in support of it has recently been presented by Hummeler and Hamparian (1958). Such common antigens could be of 2 main kinds. Antigens associated predominantly with one of the three virus types, and therefore designated " type-specific ", might be present in relatively minute amounts in one or both of the other types ; for the purpose of this discussion, such antigens will be referred to as " heterotypic ". Secondly, antigens distinct

from any recognized as " type-specific " might occur in 2 or in all 3 types of poliovirus ; such " group " antigens, as they will be termed here, would by definition be more equally distributed between the different types.

Direct agar-precipitation tests have given no indication of the presence of either " heterotypic " or " group " antigens in Type 1 virus concentrates, but immunization of rabbits with unheated Type 1 virus elicited low levels of neutralizing and precipitating activity against each of the other 2 types. No absorption tests were done, and one cannot tell whether the neutralization of Types 2 and 3 was strictly " type-specific," or whether a " group " antigen may have been involved. The precipitation, however, appeared " type-specific," judging by the character of the bands. This suggests that minute and weakly immunogenic amounts of the " heterotypic " antigens must be present, as such, in the Type 1 virus suspension, or else that the Type 1 antigen itself, at some stage after injection, must sufficiently resemble the " type-specific " antigens of the other virus types to give rise to antibody molecules which can combine with them. The serum of rabbits injected with unheated or heated Brunhilde virus gave no precipitate which could be related to any " group " antigen, as defined above.

It must be emphasized that these agar-diffusion tests have been done with immune monkey and rabbit sera. It is possible that various human sera may contain antibodies which can react with " group " antigens to form precipitates different from any hitherto observed with animal sera. Meanwhile, the present findings, as far as they go, support the earlier evidence that strains of one type of poliovirus may possess characters related to the " type-specific " components of one or both of the other 2 types (Sabin, 1952 ; Howe, 1954 ; Melnick, 1955 ; Schmidt and Lennette, 1957).

The chessboard titrations of a Brunhilde virus concentrate, before and after heating (Fig. 4), relate to two disparate antigen-antibody systems, D and C. As tested by precipitation in agar, using animal immune sera, these systems have no detectable common components which can be quantitatively compared. However, the results of the two titrations show some striking resemblances as well as apparent differences. The slopes of the serum-dilution lines and the positions of the equivalence zones are the same for both systems. Each antigen was tested against a wide range of homologous antibody concentrations : the highest reacting dilution (maximum titre) of the D antigen was 1/32 ; that of the C, 1/8. Comparative titrations of other unheated and heated virus concentrates have shown a similar difference between the maximum D and C antigen titres. These findings cannot yet be explained. Of several possibilities, perhaps the most substantial is that each D-antigenic particle becomes converted by heat into a corresponding C-antigenic particle, both kinds of particles being able to form antigen-antibody aggregates with similar physical properties. On this hypothesis, the experimental results would mean that a fraction of the C particles must have been lost, by being rendered non-precipitating or incapable of diffusing into the agar because of aggregation. That such aggregation of poliovirus particles can occur is indicated by the experiments of Roizman and his colleagues (1959), who found that heating a purified Mahoney fraction SDG^D caused some of its virus to sediment more rapidly.

Whatever may be the explanation of the difference in titre between the D antigen of the unheated and the C antigen of the heated virus concentrate, it would seem that the reactive C particles in the heated preparation have a diffusion

coefficient similar to that of the D particles in the unheated. This conclusion is based on the comparable position of the equivalence zones before and after heating (Polson, 1958), and on the assumption that the diffusion coefficients of the anti-D and anti-C antibody molecules are about equal.

SUMMARY

Poliovirus particles of each serological type exist in one or other of two type-specific antigenic forms, D or C. Some of the differences between these forms have been examined by the method of agar-diffusion, using centrifuge concentrates of tissue-culture virus and monkey or rabbit antisera. The distinct nature of the D and C configurations is shown by the non-fusion of their specific precipitates in agar, their inability to absorb each other's antibodies, and their different behaviour as immunizing antigens. Measurement of the position of precipitates in agar provides a simple and useful means of assaying the D and C antigens.

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TWO DISTINCT FORMS OF EXPERIMENTAL NEPHROCALCINOSIS IN THE RAT

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NEPHROCALCINOSIS may be defined as the deposition of calcium in the kidney. A minor degree of calcium deposition is commonly found in the kidneys of elderly rats and can be produced in young animals by various dietary deficiencies which may damage the tubules (Dick and Prior, 1951), or by an abnormal amount of phosphate in the diet (Mackay and Oliver, 1935; Friedman, 1948; Craig, 1957). Larger amounts of calcium are deposited in the kidney after treatment with Vitamin D or parathyroid extract (Harris and Moore, 1929; Duguid, Duggan and Gough, 1930; Morgan and Samisch, 1935). There have been few very detailed histological studies, but the site at which the calcium is deposited appears to vary with the different experimental procedures used. Mackay and Oliver (1935) gave rats an excess of various phosphate salts by mouth and found calcium deposits in the inner cortex, related to the terminal parts of the first proximal convoluted tubules. However MacFarlane (1941), who gave an excess of acid sodium phosphate to rats, placed the site of calcium deposition in the ascending limb of Henle's loop. Engfeldt, Gardell, Hellström, Ivemark, Rhodin and Strandh (1958) injected parathyroid extract into rats and found that initially calcium salts were deposited only in the proximal convoluted tubules, but they subsequently appeared in the lumen of tubules in the medulla. Chown, Lee, Teal and Curry (1939) gave neonatal rats parathyroid hormone or vitamin D and found that the site of calcium deposition was very variable; in some cases it was only in the cortex, and in others it was confined to the medulla. Harris and Moore (1929) treated rats with an excess of vitamin D and found extensive areas of calcification in the cortex, medulla and pelvis of the kidneys.

Since the nephrocalcinosis produced with vitamin D or parathyroid extract is presumably associated with a rise in plasma calcium, it seemed of interest to study the site of calcium deposition following the injection of a pure calcium salt. As both the vitamin and the hormone treatment may be associated with an increase in the urinary excretion of phosphate, the effect of injecting phosphate alone was also investigated. Neither calcium nor phosphate were given by mouth, since an excess of one might affect the absorption of the other from the gut.

MATERIAL AND METHOD

Male rats of the M.R.C. hooded strain, aged 10–28 weeks and weighing 150–250 g. were used. Each pair of experimental and control animals was drawn from the same litter. From the time of weaning the rats had a diet of Oxoid rat cubes containing approximately

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0·8 per cent of calcium, 0·6 per cent of phosphorus, and 1 per cent of " stabilised vitamin A and Vitamin D concentrate ".

Thirty-seven rats were given calcium gluconate, 1·5 ml. of a 10 per cent solution, intraperitoneally daily for 4 to 23 days. Each injection contained 13 mg. of calcium. Thirty rats were given sodium phosphate, 2 ml. of a Molar solution (pH 7·4), intraperitoneally on alternate days for 8–20 days. Each injection contained 46 mg. of phosphorus. The control animals were given the same volumes of physiological saline by intraperitoneal injection.

Urine was collected over 2-day periods from groups of 2–3 rats in metabolic cages.

Rats were killed by exsanguination under light ether anaesthesia and serum was separated for the estimation of calcium and phosphate. One kidney was used for chemical analysis, half for the determination of calcium and the other half either for a second determination of calcium or for the determination of phosphorus. Portions of the other kidney were embedded in paraffin after fixation either in formalin buffered with Veronal to pH 7·6 ; or in 0·4 per cent 5-amino-acridine HCl in 30 per cent alcohol (Williams and Jackson, 1955) ; or in cold 80 per cent alcohol.

Calcium was demonstrated in sections with Kernechtrot, which forms a red lake with calcium salts (McGee-Russell, 1955), and with Murexide, which forms a coloured chelate with calcium (Kaufman and Adams, 1957). The von Kossa method was also used, modified so that material staining by the periodic acid-Schiff (PAS) method could be demonstrated on the same section (Moffat, 1958). Carbonates were demonstrated in sections by Bunting's method, described by Pearse (1953). Other staining techniques included Azure A (Kramer and Windrum, 1955) for the demonstration of metachromasia, Alcian blue (Pearse, 1953), for acidic mucopolysaccharides, and a method for the demonstration of alkaline phosphatase activity and PAS positive material on the same section (Moffat, 1958).

Calcium in the tissues, urine and serum was estimated by the method of Sobel and Sobel (1939). Inorganic phosphate in the serum and urine was estimated as phosphorus by Gomori's method (1942) and in the kidneys by the method of McCance and Shipp (1933).

RESULTS

Control rats

Chemical estimations.—All the tissue analyses are expressed as mg. per g. of fat-free dry weight of tissue.

In 40 control rats the calcium content of the kidneys ranged from 0·204–0·680 mg./g. The calcium content of the heart and liver was estimated in 4 of these animals. In the heart the calcium content ranged from 0·193–0·296 mg./g. (mean 0·253 mg./g.) and in the liver from 0·086–0·194 mg./g. (mean 0·134 mg./g.) The amount of phosphorus was estimated in kidneys from 6 animals and ranged from 12·6–14·3 mg./g. (mean 13·4 mg./g.) (Table I). Table II shows the serum levels of calcium at the time of death ; the serum phosphate levels were not altered by the experimental procedures and are therefore not given in a table. The serum calcium ranged from 9·2–12·4 mg. per cent and the serum phosphate from 6·6–9·4 mg. per cent. Table III gives the urinary output of calcium and phosphorus over 24 hr.

Histology.—No calcium deposits were seen in kidney sections from the control rats and no other abnormalities were found.

Rats injected with calcium gluconate

These rats appeared healthy and gained as much weight as their controls.

Chemical estimations.—Eighteen rats were given 7–11 daily injections of calcium gluconate and killed the day after the last injection. The mean calcium content of the kidneys of these animals was 6·6 mg./g. The range was wide,

TABLE I.—*The Calcium and Phosphate Content of Kidneys on which Both These Estimations were Made*

Treatment.	Kidney Ca. mg./g. fat-free dry tissue.	Kidney P. mg./g. fat-free dry tissue.
Control	0·204	13·0
	0·236	13·6
	0·239	13·5
	0·360	14·6
	0·366	12·8
	0·376	12·6
Mean	0·297	13·3
Calcium gluconate injections	2·570	16·0
	4·620	16·2
	4·810	15·1
	5·770	17·1
	8·650	17·5
	12·100	21·2
Mean	6·42	17·2
Phosphate injections	1·730	13·3
	1·790	13·7
	2·340	15·0
	3·398	14·8
	4·600	14·7
	3·928	15·4
Mean	2·964	14·5

2·0–12·1 mg., but the values were always very much greater than in the control animals. In a further 2 groups of 4 rats each given either 4 or 23 injections the calcium content of the kidneys fell nearly within the same range. The variation in response was not related to the age or weight of the rats. The calcium content of the heart and liver was estimated in 4 rats; it was the same as in the normal rats.

EXPLANATION OF PLATES

FIG. 1.—Section of kidney from a rat injected with calcium. Red calcium lakes are seen solely in the outer strip of the cortex. Kernechtrot. × 5.

FIG. 2.—Section of kidney from a rat injected with phosphate. Red calcium lakes are seen mainly in the cortex between the inner and outer zone, but there are small deposits throughout the kidney. Kernechtrot. × 5.

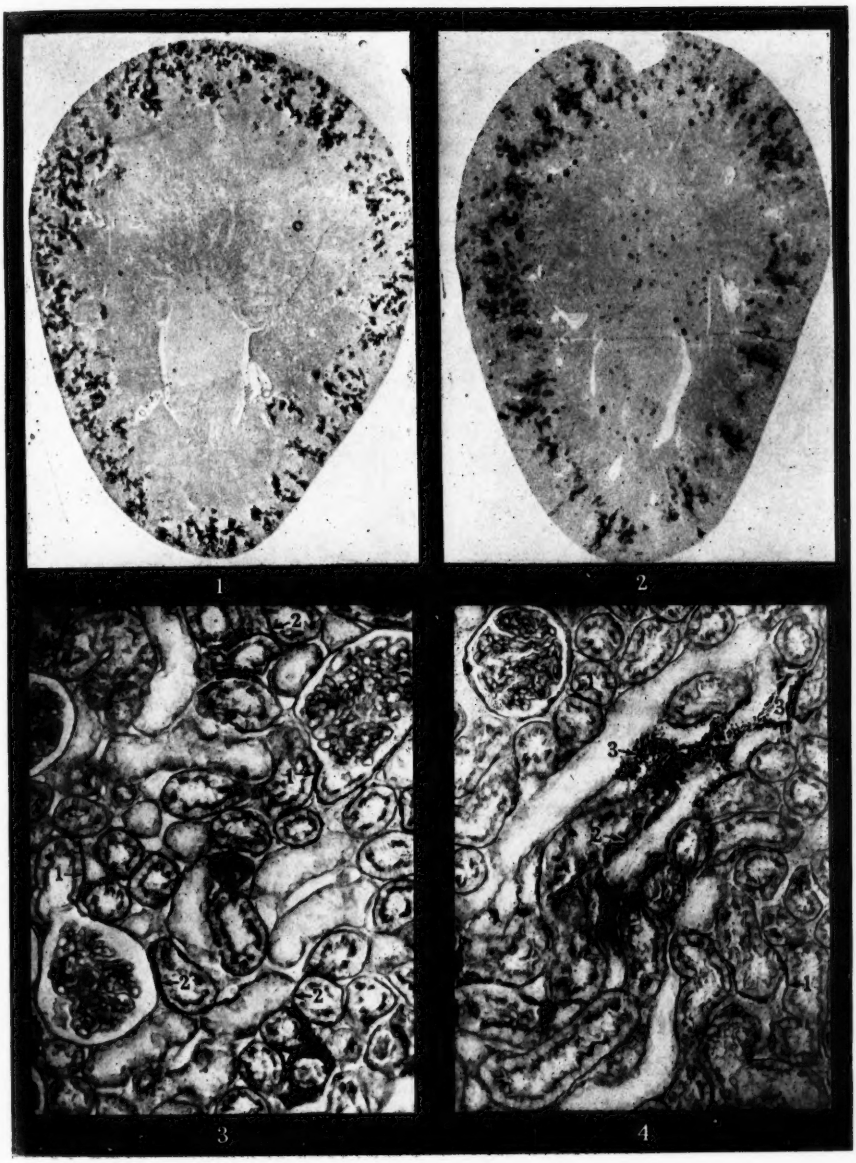
FIG. 3.—Section of kidney from a rat injected with calcium. Calcium is in the basement membrane of proximal convoluted tubules at the point at which they commence (1) and around the first convoluted part (2). The parietal layer of Bowman's capsule has no silver deposits but stains deep red. Von Kossa, PAS (Moffat). × 145.

FIG. 4.—Section of kidney from a rat injected with phosphate. Calcium is seen in the basement membrane of convoluted (1) and straight portions (2) of the proximal convoluted tubule. A coarse deposit is also seen surrounding tubules with no brush borders (3), small collecting ducts and ascending loops of Henle. von Kossa, PAS (Moffat). × 145.

FIG. 5.—Section of kidney from a rat injected with calcium gluconate, stained to show normal alkaline phosphatase activity of the brush borders (1) of tubules surrounded by calcified basement membranes (2). Moffat's stain. × 287.

FIG. 6.—Section from the kidney of a rat injected with phosphate, showing calcified casts in the medullary rays. Co-Sulphide. Azure A. × 81.

FIG. 7.—Section from a phosphate injected rat. On the left is a glomerulus with calcium deposited in it (1) and several calcified basement membranes (2). In the centre of the field is the inner zone of the cortex and the border of the medulla on the right. In these can be seen dilated tubules with thickened but uncalcified basement membrane (3). von Kossa, PAS (Moffat). × 81.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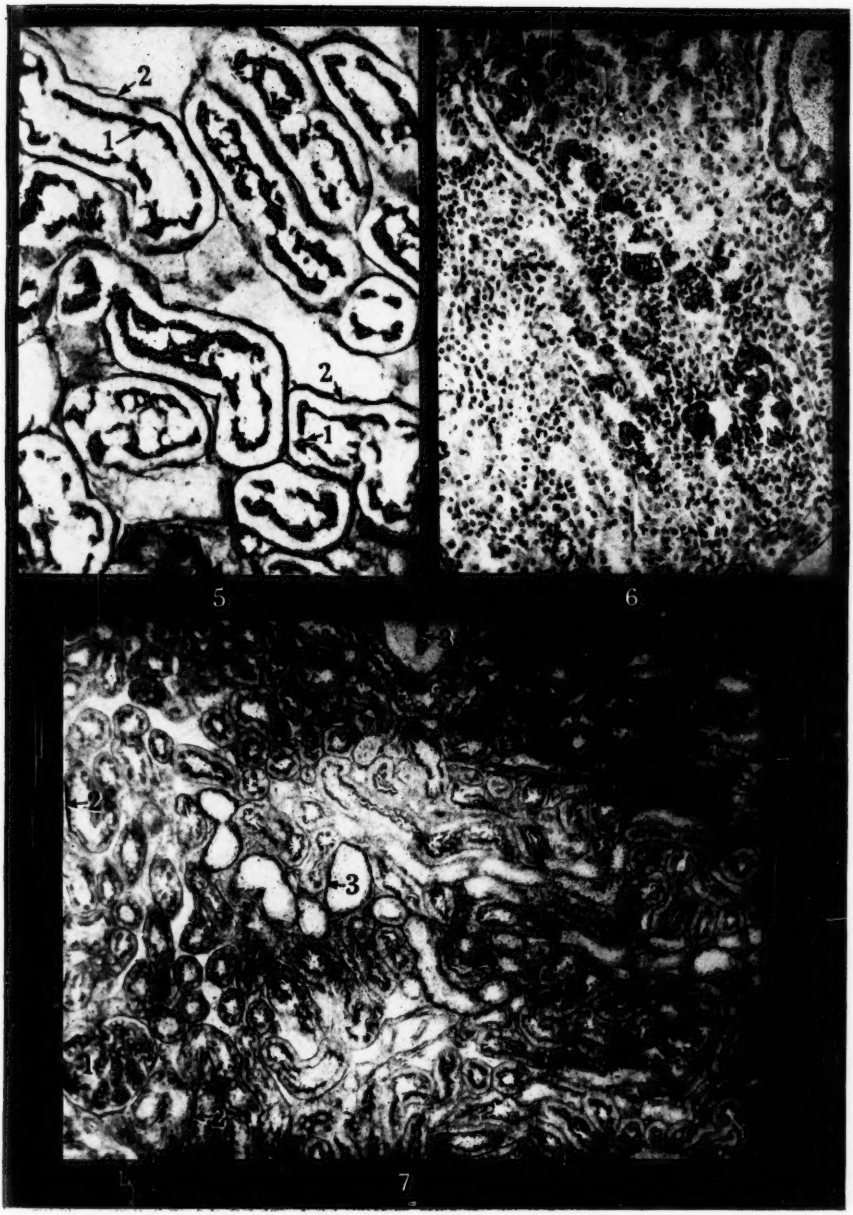
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The amount of phosphorus was estimated in the kidneys from 6 animals and ranged from 15·1–21·2 mg./g. (mean 17·2 mg./g.) (Table I).

The serum calcium level at the time of killing was greater than in the litter mate controls (Table II), but the serum phosphate was normal. The daily urinary excretion of calcium was increased, but the urinary excretion of phosphorus was less than in the controls (Table III).

Eleven rats were given 8 injections and killed 10, 24, 30 or 40 days after the last injection; the amount of calcium in their kidneys was similar to that of animals killed 24 hr. after the last injection.

TABLE II.—*The Serum Calcium of Pairs of Experimental and Control Rats*

Treatment.	No. of injections.	Ca. mg./g. fat-free dry kidney.	A Serum Ca. mg./100 ml.	B Control rat Serum Ca. mg./100 ml.	A–B Difference between serum Ca. level of injected and control rats.
Calcium	4	7·38	10·5	10·4	0·1
	5	5·43	11·4	10·1	1·3
	9	2·36	11·1	10·2	0·9
	10	4·63	13·0	9·9	3·1
	10	4·10	12·2	9·2	3·0
	10	6·86	15·8	9·7	6·1
	18	17·99	15·6	12·4	3·2
Mean		6·96	12·8	10·3	2·5
Phosphate	4	1·11	10·3	10·1	0·2
	7	1·44	10·6	9·7	0·9
	6	2·92	11·9	10·9	1·0
	6	2·31	15·3	12·4	2·9
Mean		1·94	12·0	10·8	1·2
Calcium and Phosphate	5	2·89	13·9	10·9	3·0
	10	1·27	11·9	9·7	2·2
	13	13·50	11·3	10·4	0·9
Mean		5·88	12·3	10·3	2·0

TABLE III.—*Urinary Excretion of Calcium and Phosphate. Each Estimation is Based on a Collection from 2 or 3 Rats, but the Results are Expressed as Output per Rat in 24 hr.*

Treatment.	Number of estimations.	Output of Ca. mg./rat in 24 hr. (mean).	Output of P. mg./rat in 24 hr.(mean).
Controls	4	0·423	5·05
Calcium gluconate	6	1·419	3·946
Phosphate	6	1·258	24·17
Calcium and phosphate	3	0·459	16·1

Histological changes.—Calcium deposits were demonstrated in sections of all the kidneys. The coloured calcium lakes produced with Kernechtrot were poorly localised but this method showed that the carbonates or phosphates which the von Kossa method demonstrated were in fact salts of calcium (fig. 1). The calcium was in the basement membranes surrounding tubules in the outer part of the cortex (fig. 3). None could be demonstrated in other parts of the kidney even when the chemical analysis had revealed 18 mg./g. of calcium. The portion

of the nephron round which the calcium was deposited was identified as proximal tubule by the presence of a brush border (figs. 3 and 5).

The proximal convoluted tubule of the adult rat can be divided histologically into two segments by the position, by the staining of the brush borders with the PAS technique and by its alkaline phosphatase activity. The first segment lies with the glomeruli in the outer part of the cortex ; the brush border stains pink with the PAS technique, and has only moderate alkaline phosphatase activity. The second segment lies in the inner cortex ; the brush border stains a deep red with the PAS technique and it has pronounced alkaline phosphatase activity. It was the basement membrane of the first part of the proximal tubule which was calcified (Fig. 8). Heavily calcified basement membranes were occasionally thickened. They stained brilliantly with Alcian blue and deeply with PAS but they were not metachromatic.

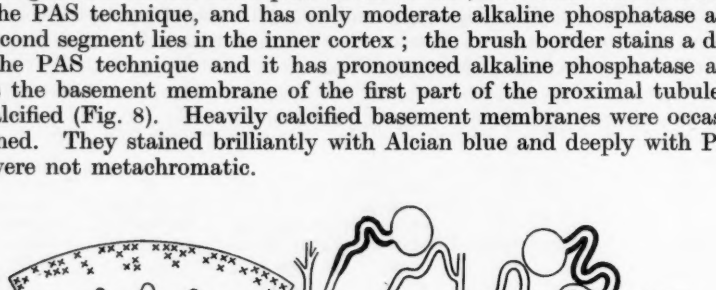

FIG. 8.—The distribution of calcium in the kidney of rats injected with calcium gluconate. Calcium solely in the basement membranes of first proximal convoluted tubules. Crosses indicate site of calcium deposits seen in section. Calcified basement membranes and intratubular casts are shown by heavy black lines.

The kidneys after formalin fixation appeared normal in sections stained by all methods other than those which demonstrate calcium or calcium salts. In preparations fixed with alcohol the brush borders of the affected tubules were well preserved and their alkaline phosphatase activity was unimpaired (Fig. 5). Amino-acridine fixation revealed that in kidneys with more than 10 mg. Ca/g. the cytoplasm of cells lying on heavily calcified basement membranes was shrunken. These preparations showed a coarse purple network in the shrunken cytoplasm when stained with Azure A.

The histological appearance of the kidneys from rats killed at different intervals after the last injection were the same. The calcium deposits remained and there was no evidence that they led to any progressive changes.

Rats injected with sodium phosphate

All of these 30 rats failed to gain as much weight as their controls. Four of them died before the end of the planned experimental period. In 2, blood was found in the thoracic cavity.

Chemical estimations.—Nineteen rats were given 6–10 injections of phosphate solution on alternate days and killed the day after the last injection. In 18 rats the mean calcium content of the kidneys was 2·8 mg./g. (range 1·6–4·6 mg./g. The remaining rat kidney had an exceptionally high content of calcium—26·6 mg./g.).

Four rats were given only 4 injections; in 2 of these the calcium content of the kidney was normal and in the other 2 the increase in calcium content was relatively small, 1·1 and 1·4 mg./g. The calcium content of the heart and liver was estimated in 4 rats. It was the same as in the normal rats.

The phosphorus content of the kidneys was measured in 6 rats and ranged from 13·3–15·4 mg./g. (mean 14·5 mg./g.) (Table I).

The serum calcium level varied from 10·6–15·3 mg. per cent (Table II). The serum phosphate level was not raised. The daily urinary excretion of phosphorus was increased; there was only a small increase in urinary calcium (Table III).

Seven rats were given 10 injections of phosphate and killed 2 days, 4, 5 or 6 weeks after the last injection. The amount of calcium in the kidneys did not diminish appreciably.

Histological changes.—Calcium could be demonstrated histologically in sections of kidneys from two of the four rats that had received 4 injections of phosphate solution and in sections from all that had been given a greater number of injections. The red lakes formed with Kernechtrot and the binding of Murexide showed that deposits revealed by the von Kossa method consisted of calcium salts (Fig. 2). The presence of carbonates associated with the calcium was demonstrated. The site of calcium deposition varied but in most cases it was mainly in the cortex. In kidneys with minimal deposits the calcium was only in the basement membrane of radiating tubules in the cortex (Fig. 4). These represent terminal straight parts of proximal convoluted tubules, ascending loops of Henle and small collecting ducts. In more heavily calcified kidneys there was in addition calcium in the basement membrane of the first part of the proximal convoluted tubule as in the animals treated with calcium gluconate. In about half the kidneys there were flecks of calcium in the glomerular basement membrane and there were also small intratubular casts containing calcium in the cortex and medulla (Fig. 9). In the kidney which had 26·6 mg./g. of calcium the deposit was predominantly in the form of intratubular casts which were shown by Bunting's method to contain carbonates. The casts were mainly in the medullary rays of the cortex (Fig. 6).

In addition to calcification other abnormal features were found in 22 of the 30 kidneys from phosphate treated rats. The commonest were a localised breakdown of brush borders with loss of alkaline phosphatase activity and, in the medulla, casts which stained deep red with PAS, but contained no calcium; some of these casts showed alkaline phosphatase activity, confirming Duguid's (1936) suggestion that they were made up of broken off brush borders. In half the kidneys groups of tubules in the cortex and medulla were dilated (Fig. 7); their basement membrane was much thickened but in most cases not calcified. More than a third of the kidneys from these animals had areas of round cell infiltration which usually surrounded blood vessels at the cortico-medullary junction; round cell infiltration was not found in control sections. All these abnormalities were more pronounced in younger animals. There did not appear to be any direct relation between these abnormalities and the amount of calcium

in the kidneys, but the two kidneys which contained normal amounts of calcium were histologically normal. The kidneys of animals killed at varying intervals after the last phosphate injection showed the same histological picture as those killed after 24 hr.

Rats injected with both calcium gluconate and sodium phosphate

Three rats were given intraperitoneal injections of calcium and of phosphate (5, 10 or 13 injections of each) on alternate days. As in the other experiments the amount of calcium deposited in the kidney (2·9, 1·28 and 13·5 mg./g.) was not directly related to the number of injections. The serum phosphate was normal but the serum calcium was raised (Table II). The excretion of phosphorus in urine was increased (Table III), but the excretion of calcium was normal.

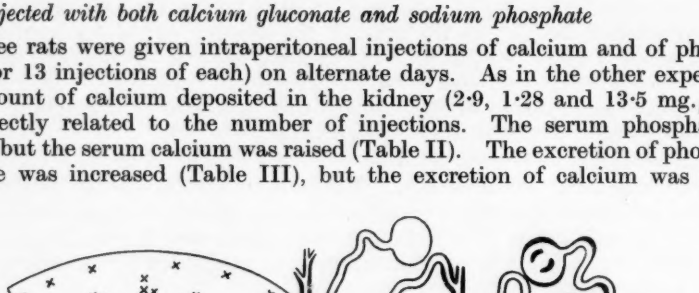

FIG. 9.—The distribution of calcium in the kidney of rats injected with sodium phosphate. Calcium in the basement membrane of all segments of the proximal convoluted tubules, of the glomeruli, of the ascending limbs of Henle's loop and of the small collecting ducts. Intratubular casts in the collecting ducts. Crosses indicate site of calcium deposits seen in section. Calcified basement membranes and intratubular casts are shown by heavy black lines.

The histological picture was a combination of that seen in rats injected with calcium alone, with that seen in rats injected with phosphate alone. Calcium was deposited in the basement membrane of the first part of the proximal convoluted tubule as in rats given calcium injections, and around other segments of the nephron and in the form of intratubular casts as in phosphate treated rats. The kidneys were damaged as in rats given phosphate alone.

DISCUSSION

Calcium gluconate has also been given to rats by Baker, Reaven and Sawyer (1954) and by Grimes (1957) in the same daily dose as we have used. Baker *et al.* could not demonstrate calcium in the kidneys of rats given 28 daily injections, although the mean serum calcium of the rats was 13·6 mg. per cent. The technique of Baker *et al.* differed from ours in several ways, notably in that they used unbuffered formalin. We have found that fixation even in neutral formalin

buffered with phosphates removes calcium deposits from the basement membrane of the nephron. Grimes (1957), using von Kossa's method, demonstrated calcification in the basement membranes of the proximal convoluted tubules and of the parietal layer of Bowman's capsule after 14 injections. We have not seen calcium in the glomerular basement membrane of rats injected with calcium gluconate.

The injection of calcium gluconate raised the serum calcium and therefore presumably increased the amount of calcium filtered through the glomeruli. The increase in the urinary calcium was less than a tenth of the amount injected and hence the amount of calcium reabsorbed by the tubules had increased. Calcium was deposited in the basement membrane of a strictly defined segment of the nephron. It is not known in which part of the tubule calcium is reabsorbed, but the localisation of the calcium deposit to the first part of the proximal convoluted tubule may mean that this is the site of its reabsorption.

Duguid (1936) injected mono-basic sodium phosphate into rats and produced different histological results from ours. He concluded that the whole of the proximal convoluted tubule was affected and that there was no evidence of selective action on any particular part of it, although the terminal segments tended to become packed with solid material derived from higher up and this became calcified. His results differed from ours either because he gave a larger dose of phosphate to younger rats or because he gave the acidic salt.

In our experiments the phosphate injections had a variable effect on the serum calcium levels and presumably did not always increase the amount of calcium filtered by the kidneys. There was however an increased urinary excretion of calcium as well as of phosphorus. In the kidneys calcium was deposited around segments of the nephron which were not affected by calcium gluconate injections. This could be explained by the formation of calcium phosphate and its subsequent passage through the renal tubule, but histochemical evidence shows that the calcium casts are not solely in the form of phosphate but of carbonate as well. When we injected both calcium and phosphate the histological picture was as could have been predicted from the combined effect of the two types of injections and showed no new features.

Apart from the variation in the site of calcium deposits the most noticeable difference between the kidneys of the phosphate and calcium injected animals was the histological evidence of damage after phosphate injections compared with the surprisingly normal appearance after calcium injections. This difference was reflected in the general state of health of the 2 groups of animals. Mackay and Oliver (1935) injected phosphate into young rats (4–10 weeks) and found a "transient" swelling of the renal tubular epithelium, suggesting a toxic effect of phosphate on the renal cells, but found no deposits of calcium. We assume that the kidneys in our rats were damaged by the excess of phosphate passing through them and calcium was deposited in the damaged tissues.

In clinical and experimental hyperparathyroidism, in hypervitaminosis D and in osteolytic conditions, the excretion of phosphorus as well as of calcium is increased. This may account for the fact that the strictly localised type of deposition of calcium produced in the kidney by calcium gluconate injections is not seen in other types of nephrocalcinosis. The results obtained in rats with parathyroid hormone, with Vitamin D, or by altering the calcium phosphorus ratio of the diet resemble those that we have obtained with phosphate injections.

SUMMARY

The daily intraperitoneal injection of 13 mg. of calcium as the gluconate into rats raised the serum calcium and increased the urinary excretion of calcium. The calcium content of the kidneys increased and ranged from 2·0–18 mg./g. (normal mean 0·3 mg./g.). This variation could not be related to the amount of calcium injected or to the weight or the age of the rats. The phosphorus content of the kidneys was not raised in proportion; the mean value was 17·2 mg./g. of fat-free dry kidney (normal mean 13·4 mg./g.).

The calcium was deposited in the basement membrane of the first part of the proximal convoluted tubules and nowhere else. In rats killed up to 40 days after the last injection comparable amounts of calcium remained in the kidney at the same site. Apart from calcium deposits the kidneys showed little histological abnormality.

The intraperitoneal injection on alternate days of 46 mg. of phosphorus given in a sodium phosphate solution (pH 7·4) did not raise the serum phosphate in rats, but increased the excretion of phosphorus and calcium in the urine. In all but two of the rats given phosphate calcium was deposited in the kidneys. The calcium content ranged from 1·1–26·6 mg./g. of fat-free dry kidney and depended on the number of injections given. The phosphorus content of the kidneys was raised; the mean value was 14·5 mg./g. of fat-free dry kidney. The site of the calcium deposition in the kidney varied, but in most kidneys it was mainly in the cortex. The calcium appeared first in the basement membranes of the medullary rays, representing a segment of the second part of the first proximal convoluted tubules, the ascending limb of Henle's loop and the small collecting ducts; later it appeared in the glomerular basement membrane and the first part of the proximal convoluted tubule. Casts, some of them calcified, were often seen inside the tubules of the medulla; more than half the kidneys showed other pathological changes. In rats killed up to 6 weeks after the last injection similar amounts of calcium remained in the kidneys and the histological picture had not changed.

The calcium content of the heart and liver was estimated in four rats from each group and found to be the same as in four control animals.

The nephrocalcinosis produced by calcium gluconate injections may be the result of the increase in the calcium content of the serum and the urine. The nephrocalcinosis produced by phosphate injections may be secondary to renal damage induced by a large excretion of phosphate.

I should like to express my thanks to Professor J. S. Baxter and to Professor H. Scarborough for their interest and encouragement and to Dr. P. Fourman for his advice and help in this work. It was carried out with the aid of a grant to Dr. P. Fourman from the Empire Rheumatism Council. I am indebted to Miss V. Mahoney, Mrs. A. Robinson and Mr. M. Morgan for technical assistance and to Mr. A. Welch for the microphotographs.

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POSSIBLE FACTORS IN THE PATHOGENESIS OF CHOLERA

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CHOLERA is characterised by a great and sudden loss of fluid and salts from the intestine. Since *Vibrio cholerae* is confined to the lumen of the intestine and has been isolated only on very rare occasions from other parts of the body, it seems likely that the most important factors involved in the pathogenesis of the disease are those which cause gross changes in the permeability of the small intestine.

There have been reports of two different substances which may be implicated in these permeability changes. Burrows, Wagner and Mather (1944) claimed that a small dialysable toxin isolated by Burrows (1944) from *V. cholerae* caused changes in the permeability of rabbit intestine to water, *in vitro*. Burnet and Stone (1947) found that cultures of *V. cholerae* contained a mucinase enzyme distinct from the receptor destroying enzyme which caused desquamation of the lining epithelium of strips of guinea pig ileum. It has been further claimed that *V. cholerae* filtrates rich in mucinase activity alter the permeability of mouse small intestine to water, *in vivo* (Lam, Mandle and Goodner, 1955).

Due to the lack of an experimental animal in which the typical symptoms of human cholera can be reproduced, the significance of these factors in the pathogenesis of the disease is still obscure. In 1953 De and Chatterjee showed that cultures of *V. cholerae* injected into ligated loops of rabbit small intestine, resulted in a distension of the loops with fluid, and desquamation of the lining epithelium with haemorrhagic necrosis of the wall. This experimental technique offers a method by which bacterial fractions may be examined for their ability to reproduce similar changes to those seen when living cultures are injected.

In this paper, various toxic fractions isolated from cultures of *V. cholerae* (Jenkin and Rowley, 1959) have been tested by this technique, bearing in mind the possibility that the same determinant factors might be operating in this experimental test as in cases of human cholera.

MATERIAL AND METHODS

Bacterial strains.—The strains of *V. cholerae*, Ogawa 5596, Inaba 4693, and Hikojima 7270 were obtained from the National Collection of Type cultures. *Bacterium coli* strains 3884, 2712, 8004 and 2821 were kindly supplied by Dr. Roberts, Department of Pathology, Ruchill Hospital, Glasgow. The *Staphylococcus aureus* 524 was obtained from Dr. Rogers of the National Institute of Medical Research, Mill Hill.

Operative procedure.—A light anaesthesia was induced in the rabbit by intravenous injection of 0·7 ml. of a 5 per cent solution of sodium thiopentone. A small area of the abdomen was shaved and a deeper surgical anaesthesia induced with ether. With a scalpel a shallow incision about 2 in. long was made, slightly to the right of the mid line and the subcutaneous layers exposed. The skin flaps were dissected off and held apart by Spencer Wells artery forceps. A further incision was made, about 1 in. in length, through the muscle layers to the peritoneum. The peritoneum was opened with small scissors and the gut exposed. The

appendix was located manually and a loop of the ileum carefully withdrawn. Warm saline packs were placed around the incision covering the whole of the abdomen. About 3 ft. of the ileum was taken out and covered with warm saline packs, except for the small length chosen for the first experimental loop. Two ligatures were made approximately 3 in. apart with strong cotton, care being taken that none of the blood vessels supplying the loop were damaged or tied off. Coloured threads were used so that a particular loop could be easily identified. The cultures or fractions under test were introduced into the lumen of the ligated loop with a sterile syringe fitted with a gauge 22 needle and the loop then pushed back into the peritoneal cavity. In each animal 3–4 loops were made separated by at least 6 in. After tying off the loops and replacing the intestine, the muscle layers were sewn up with nylon thread, using a running stitch. The skin layer was sutured with 3–4 Michel clips and the abdomen gently manipulated to re-distribute the small intestine. The whole operation took 15–20 min. Most rabbits were making attempts to stand within 1 hr. and were always standing within 2 hr.

Consistent experimental results were obtained providing that young rabbits 12–24 weeks old were used and that the loops were situated in the ileum. The duodenum gave variable results but the ileum appeared to be more sensitive and gave consistent results. The strain of rabbit seems to be immaterial; six different breeds have been successfully used. The rabbits were always starved 24 hr. beforehand, but allowed water.

Preparation of cultures for injection.—This was found by experience to be the most important factor in ensuring consistent results. *V. cholerae* cultures were grown at pH 8·2 in nutrient broth, whilst *Bact. coli* strains and the *Staph. aureus* 524 were grown at pH 7·4. The cultures were planted in 200 ml. of broth in Erlenmeyer flasks and shaken to ensure good aeration at 37° overnight. The following morning a hundred-fold dilution of the culture was made in broth and shaken as before for a further 2 hr. at 37°. During the operation the culture was kept at 37° in a waterbath. These precautions ensured that a growing culture was injected into the loop. The pH of the broth appeared to be immaterial providing the strain grew well at that particular pH. All three cholera serotypes gave positive loops. (Fig. 1).

Bacterial counts on positive and negative loops.—Positive and negative loops were carefully removed and washed in sterile saline. The ligated loop was placed in a sterile petri dish, the ligatures removed and the contents washed out with sterile saline. Serial dilutions of the washout were made in saline and plated out on MacConkeys agar. After overnight incubation a hundred or more colonies selected at random were tested against a 1/500 dilution of the homologous antiserum. By this means an estimate of the total number of viable organisms of the specific type could be obtained.

Preparation of toxic fractions.—The protein toxin and lipopolysaccharide endotoxin were prepared as described by Jenkin and Rowley (1959).

Mucinase preparation and assay.—Filtrates containing high titre mucinase were prepared by the method of Chugh, Jensen and Kendrick (1956) and assayed according to Burnet (1949). For use in experiments with crude intestinal mucin, the filtrate was dialysed against 0·1 M phosphate buffer pH 7·3 containing 1 per cent $CaCl_2$.

Preparation of crude intestinal mucin.—The small intestine from rabbits which had been starved for 24 hr. was washed through with 0·1 M phosphate buffer at pH 7·3. It was then opened along its length and cut into a number of small segments which were suspended in the phosphate buffer washings and incubated at 37° with shaking for 2 hr. The suspension was centrifuged at 3,000 *g.* for 20 min. and the resulting supernatant precipitated with five volumes of absolute alcohol. The precipitate removed by centrifuging was dissolved in 0·1 M phosphate buffer pH 7·3 containing 1 per cent $CaCl_2$ and dialysed against the same with frequent changes for 48 hr. at 0°.

Estimation of Lactic acid.—Lactic acid was estimated by the method of Elsden and Gibson (1954).

Estimation of Hexosamine.—Hexosamine was estimated by the method of Rondle and Morgan (1955).

RESULTS

In a previous paper (1959) we reported our inability to isolate a small molecular toxin by the method used by Burrows (1944). Using his experimental techniques,

alcohol soluble, but non-toxic, fractions were screened for their ability to increase the permeability of rabbit intestine to water. The experimental results were negative; no increase in the permeability of the intestinal segments to water was ever observed.

The action of toxins and enzymes from V. cholerae *on ligated loops of rabbit ileum*

The lipopolysaccharide endotoxin and protein toxin were tested in ligated loops to see if they would produce a similar fluid distension to that observed when living cultures were injected. The amounts varied from 1–10 mg. per loop. The results obtained in these experiments were negative. Combination of the two fractions with a filtrate containing 500–1,000 uts/mucinase per ml. did not produce any changes in the ligated loop of ileum. Injecting large amounts of vibrio cells (20–40 mg. per loop) killed by heating at 75° for 30 min. or by the addition of 500 μg. streptomycin/ml. again gave negative results.

Effect of injecting fluid from an infected loop into a fresh loop

It is possible that *V. cholerae* like the anthrax bacillus (Smith, Keppie and Stanley, 1955) produces a substance which causes the pathological changes seen in the ileum, only under conditions of *in vivo* growth. One might expect to find such a toxin in the fluid of an infected loop. The loop fluid contains large amounts of the mucinase enzyme 1,000–2,000 uts/ml. as well as a heavy growth of *V. cholerae*, so that all the factors which might cause damage and alteration of the permeability of the ileum are likely to be present. The fluid contents of three positive loops were withdrawn and 1 mg. streptomycin/ml. added to prevent further bacterial growth. This material was injected in 5 ml. amounts into 2 ligated loops in each of 3 other rabbits within 2 hr. after removal. The animals were killed 24 hr. later and once again all loops were negative. From these results one may conclude that none of the toxins or enzymes present in the loop fluid produced by *V. cholerae* singly or in combination could be responsible for the pathological changes in the loops.

Since positive results were obtained only in the presence of a growing culture, various simple metabolic products which might be present during growth of the organism were tested for their ability to produce a positive loop.

Effect of sodium salts of organic acids

Sodium salts of a number of organic acids such as acetic, succinic, propionic, pyruvic and lactic which are known to be breakdown products of bacterial glycolysis, were injected in various quantities into experimental loops. It was found that only in those loops injected with 20 mg. or more of sodium lactate were positive results obtained (Table I). The gross appearance of these postive loops closely resembled that seen in loops injected with living cultures. Distension with fluid was marked, haemorrhagic necrosis of the intestinal epithelium had occurred and in the majority of these positive loops sloughing of the lining epithelium was apparent.

Growth of injected bacteria in the ligated loop and production of lactic acid

Since various people (De, Bhattacharya and Sarkar, 1956; Taylor, Maltby and Payne, 1958 and McNaught and Roberts, 1958) have reported that some

Bact. coli strains will also cause changes in ligated loops of rabbit intestine similar to those seen when *V. cholerae* is injected, a number of *Bact. coli* strains, a *Staph. aureus* 524 and *V. cholerae* Ogawa 5596, were tested in ligated loops. In all cases, positive and negative loops were analysed for lactic acid content and the number

TABLE I.—*Effect of Injecting Sodium Salts of Organic Acids into Ligated Loops of Rabbit Ileum*

	50 mg.	25 mg.	12 mg.
Sodium lactate	+++	+++	—
Sodium pyruvate	—	—	—
Sodium propionate	—	—	—
Sodiu acetate	—	—	—
Sodium succinate	—	—	—

+++ fluid distension of the loop with haemorrhagic necrosis of the epithelium.

of specific bacteria determined in each loop. The different results obtained between positive and negative loops were striking. In all the positive loops the specific bacterial count was very high and the loop fluid contained significant amounts of lactic acid. In the negative loops, little or no growth had taken place and much smaller quantities of lactic acid could be demonstrated (Table II). These results confirm what had been previously found, that only in the presence of growing cultures could positive loops be obtained.

TABLE II.—*Specific Bacterial Count and Lactic Acid Content of Positive and Negative Loops of Rabbit Ileum.* 10^8 *Bacteria Injected into Each Loop.*

Strain	Total specific bacteria in loop	Lactic acid mg./loop	State of Loop
V. cholerae Ogawa 5596	2×10^{11}	27·4	+++*
	$4{\cdot}5\times10^{11}$	24·2	+++
Bact. coli 8004	$2{\cdot}8\times10^{10}$	31	+++
	$3{\cdot}2\times10^{10}$	41·5	+++
3884	$3{\cdot}0\times10^{10}$	24·3	+++
Staph. aureus 524	$5{\cdot}5\times10^{9}$	23·4	+++
Bact. coli 2712	5×10^{8}	7·0	—
	$2{\cdot}25\times10^{8}$	2·5	—
2821	$3{\cdot}2\times10^{8}$	8·4	—
	$2{\cdot}8\times10^{8}$	5·8	—

* Key as in Table III.

The role of mucinase enzyme and lipopolysaccharide endotoxin

During bacterial growth both mucinase and endotoxin will be released, the latter due to cell lysis. It seemed important to test whether these substances whilst being individually incapable of producing a positive loop, might "sensitize" the intestine to the action of sodium lactate.

Absorption of endotoxin from ligated loops

Two ligated loops in each of 4 rabbits were injected with 1 ml. of a rapidly growing culture of *V. cholerae*. After 18 hr., 2 of the rabbits presumed to contain

positive loops were given 25 μg. of lipopolysaccharide from the same organism intravenously; the other 2 animals were given 1 ml. of pyrogen-free saline. When killed 7 hr. later, the presence of positive loops was confirmed and a bilateral haemorrhagic necrosis of the kidney cortex, the beginning of the generalised Schwartzman phenomenon, could be seen in the rabbits given endotoxin whilst the controls were negative. This suggests that bacterial endotoxin has been absorbed from the gut.

Increase in sensitivity to sodium lactate in endotoxin treated rabbits

Two rabbits were given intravenously 1 ml. of saline containing 25 μg. of Ogawa 5596 lipopolysaccharide. A third rabbit acting as a control was given 1 ml. of saline only. Three hours later ligated loops were prepared in all the animals and injected with varying amounts of sodium lactate. The rabbits were killed 6 hr. later and it was found that amounts of sodium lactate gave positive loops in the endotoxin treated animals which in the control animal failed to produce any effect (Table III).

TABLE III.—*Increase in Sensitivity to Sodium Lactate in Rabbits given 25 μg. Endotoxin i.v. 3 hr. before Operation.*

Rabbit	Amount of sodium lactate injected		
	25 mg.	10 mg.	5 mg.
a	+++	++	+
b	+++	++	++
c*	+++	−	−

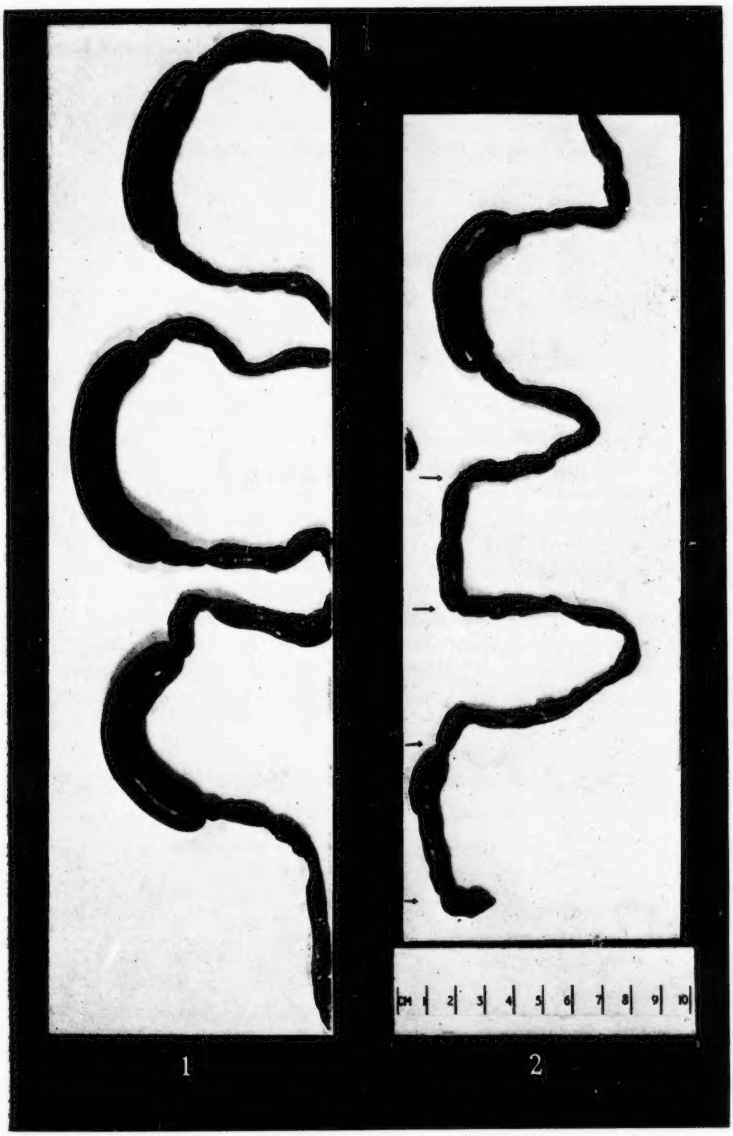
c* control animal injected with saline only.
+++ fluid distension of the loop and haemorrhagic necrosis of the wall.
++ fluid distension of the loop, few scattered haemorrhages.
+ fluid in loop, not distended and no haemorrhagic changes.

Role of mucinase

The initial studies by Burnet *et al.* (1947) on the desquamating action of the mucinase enzyme on the intestinal mucosa has been independently confirmed by a number of research workers (Singer, Wei and Hoa, 1948; Gurkipal Singh and Ahuja, 1953). However, no quantitative studies have been made on the rate of splitting of intestinal mucin by the mucinase enzyme. The assay of the mucinase enzyme using ovomucin as the artificial substrate is unsatisfactory, as it may bear little relationship to the splitting of the natural substrate. Crude intestinal mucin was prepared as described and the action of a *V. cholerae* culture filtrate on this substrate was studied by following the release of hexosamine. The results given in Table IV show that at pH 7·4, 30 per cent of the hexosamine in the substrate had been released whilst at pH 6·4 and pH 8·4 the amount of amino sugar liberated was much less. These preliminary studies suggest that the optimum

EXPLANATION OF PLATE.

FIG. 1.—Positive loops of rabbit ileum 24 hr. after injection of *V. cholerae*.
FIG. 2.—The effect of aluminium oxide on the production of positive loops by *V. cholerae*. The 2 loops at the bottom received 1 g. aluminium oxide in addition to *V. cholerae*.



Jenkin and Rowley.

activity of the enzyme lies between pH 7·0 and pH 8·0. The pH of the fluid from positive loops varied between pH 7·4 and pH 8·0, and would thus give optimum conditions for this enzymic activity.

TABLE IV.—*Release of Hexosamine from Crude Intestinal Mucin by an Enzyme Filtrate from* V. cholerae.

Time in hr. at 37°	Per cent of total hexosamine released		
	pH 6·4	pH 7·4	pH 8·4
1	0	7	0
2	7	11	0
3	14	14	0
4	14	18	7
5	14	30	7

Effect of adding adsorbents to the loops injected with V. cholerae 5596

If the mucinase enzyme and/or the lipolysaccharide endotoxin could be removed then it might be possible to prevent damage to the injected loop, the lactic acid being removed as rapidly as it is formed under relatively normal physiological conditions. A number of adsorbents such as kaolin, magnesium trisilicate, aluminium phosphate and aluminium oxide in 1 g. amounts were introduced into the ligated loop immediately after injecting 1 ml. of the growing culture. The animals were killed 24 hr. later and it was found that those loops containing aluminium oxide were negative. Control loops injected with *V. cholerae* alone were all positive (Fig. 2). The other adsorbents did not seem to have any effect in preventing a positive loop. Counts made on the aluminium oxide loops and control loops showed that the organism had grown to the same extent in each. (Table V).

TABLE V.—*The Effect of Aluminium Oxide on the Production of Positive Loops of Rabbit Ileum by* V. cholerae

Rabbit	Total *V. cholerae* in loop at time of death	ml. of fluid in loop
All loops injected with 5×10^8 *V. cholerae*.		
a	$2 \times 10^{10} \times 8$	8
b	$3 \times 10^{10} \times 12$	12
c	$2 \times 10^{10} \times 30$	30
d	$4 \times 10^{10} \times 15$	15
Loops receiving 1 g. of aluminium oxide in addition to bacteria.		
a	2×10^{10}	0
b	2×10^{10}	0
c	7×10^{10}	0
d	2×10^{10}	0

DISCUSSION

In the present study three factors have been implicated in the production of a positive loop, these are lactic acid, bacterial endotoxin and a mucinase enzyme.

If one can draw any analogy between the ligated loops and human cholera, it seems possible that these three substances may play an important role in the aetiology of this and similar enteric diseases.

From the literature it is apparent that a wide variety of bacteria both Gram-negative and Gram-positive once established in the gut may give rise to diarrhoeal enteritis. De *et al.* (1956) have reported the isolation of *Bact. coli* strains from cases that had been clinically diagnosed as cholera, whilst from many cases of cholera non-agglutinable vibrios have been isolated (Taylor, 1938). Pseudo-membranous enterocolitis, a condition in which rice-water stools are frequently passed, may be caused by *Staphylococci* (Gardner, 1953) or by *Clostridium welchii* type D (Brumfitt and Wright, 1957). The *Bact. coli* strains isolated by De *et al.* (1956) were found to cause damage to ligated loops of rabbit intestine, similar to that seen of *V. cholerae*. The ability of some *Bact. coli* strains to produce positive loops has been confirmed by Taylor *et al.* (1958) and McNaught and Roberts (1958). It is apparent from their results that there is no correlation between the serotype of the organism and its ability to produce the fluid distension and haemorrhagic necrosis.

The causation of enteric diseases by a variety of antigenically dissimilar organisms suggests that a common factor of metabolism may be involved. It is unlikely that these different bacteria possess the same endotoxins, though the mucinase enzyme appears to be widespread (McClean, 1936) so that one must look for a factor produced by these bacteria during growth. It has been shown in the present work that lactic acid or sodium lactate, if injected into ligated loops, causes fluid distension and a haemorrhagic necrosis of the epithelium. Lactic acid has been implicated in the tissue damage seen in the local and generalised Schwartzman reaction and the role it plays discussed by Thomas and Stetson (1949) and Stetson (1951). It is possible that the haemorrhagic necrosis seen in the ligated loops may be brought about by a similar mechanism. The work of Bullen and Scarisbrick (1957) on enterotoxaemia in sheep suggests that lactic acid production may be involved in altering the gut permeability thus allowing absorption of the ϵ toxin of *Cl. welchii* type D. In order to establish a toxaemia in the sheep the organism had to be multiplying rapidly in the gut of an animal on a rich carbohydrate diet. In the present study, all the bacteria giving rise to positive loops were capable of multiplying rapidly in the gut and producing lactic acid. Bacteria that had no apparent pathogenic effect multiplied slowly or not at all and only very small amounts of lactic acid were produced. From the present work a tentative hypothesis may be advanced as to the sequence of events giving rise to a positive loop. Growth of the bacteria in the loop produces concurrently mucinase, sodium lactate and bacterial endotoxin by autolysis. The mucinase enzyme destroys the intestinal mucin exposing the epithelium to the action of sodium lactate and allowing absorption of the endotoxin. Lipopolysaccharide endotoxin is known to alter the carbohydrate metabolism of the rabbit, such that there is a rise in the circulating lactic acid and accumulation of this substance in the tissues (Kun & Miller, 1948). The accumulation of sodium lactate causes tissue damage leading to gross changes in the permeability of the gut and subsequent fluid loss.

The protection afforded by aluminium oxide may be due to the absorption of mucinase enzyme and/or endotoxin, thus allowing the lactic acid produced to be taken up from the gut and broken down normally.

SUMMARY

A study has been made of factors which may be implicated in the pathogenesis of cholera and similar enteric disease using the ligated rabbit intestinal loop technique of De and Chatterjee (1953). Three factors have been found, lactic acid, mucinase enzyme and lipopolysaccharide endotoxin, the most important of these being production of lactic acid by the bacteria during growth. The possible role of these three factors in the pathogenesis of cholera is discussed.

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TAMe ESTERASE ACTIVITY OF GUINEA PIG SERUM

I. THE PLASMIN SYSTEM

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THE fibrinolytic activity appearing in serum and plasma under a variety of circumstances has been attributed to a proteolytic enzyme, plasmin. An enormous literature has grown up on the nature of this enzyme and on its possible implication in various physiological and pathological states; this has been reviewed by Macfarlane and Biggs (1948) and by Astrup (1956). Plasmin normally exists in plasma in an inactive precursor form, plasminogen. In human serum the precursor can be activated by streptokinase (SK) alone (Christensen, 1945), whereas in a number of other species a "proactivator", present in human serum, is required as well as SK. Norman (1957) and Meyers and Burdon (1957) have demonstrated that the activation of guinea-pig plasminogen requires both SK and the "proactivator" of human sera. Troll, Sherry and Wachman (1954) have shown that human plasmin, like trypsin, is capable of splitting synthetic substrates such as *p*-toluene sulfonyl L-arginine methyl ester (TAMe) and L-lysine ethyl ester (LEe) as well as the more conventional protein substrates. Moreover, it was subsequently demonstrated that the caseinolytic activity of human plasmin (Troll and Sherry, 1955) or guinea pig plasmin (Meyers and Burdon, 1957) was competitively inhibited by the presence of these amino acid esters. Treatment of animal and human serum with chloroform (Tagnon, Davidson and Taylor, 1943; Christensen, 1946; Roche e Silva and Rimington, 1948; Geiger, 1952; Loomis, 1952) or peptone (Roche e Silva, Andrade and Teixeira, 1946; Ungar and Mist, 1949; Ungar, Damgaard and Hummel, 1953) has been shown to induce fibrinolytic and caseinolytic activity.

Our interest in plasmin stems from reports that proteolytic activity appears in the serum during the course of *in vivo* anaphylactic (Lowell, Franklin, Schiller and Follensby, 1956) and anaphylactoid (Roche e Silva *et al.*, 1946) reactions and *in vitro* immune reactions (Ungar *et al.*, 1953). This observation led a number of investigators to assign a causal role to serum protease in anaphylaxis (Roche e Silva *et al.*, 1946; Ungar *et al.*, 1953), whereas others doubt its presence (Jemski, Flick and Stinebring, 1953) or significance (Dale, 1929; Geiger, 1952; McIntire, 1956; Mongar and Schild, 1957). Since the substrates employed, casein, fibrin or gelatin, are attacked by a variety of proteolytic enzymes, it is likely that various investigators were studying different enzyme systems, either single or in combination. By the use of a variety of synthetic substrates such as TAMe LEe, N-acetyl L-tyrosine ethyl ester (ATEe) and others a better separation of enzyme systems seemed possible. Accordingly, a study was undertaken of the

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effect of the *in vitro* antigen-antibody reaction on the TAMe esterase activity of guinea pig serum, with the hope of evaluating the role of plasmin or other enzyme systems in the anaphylactic reaction.

These studies were carried out on serum rather than on some purified product, to avoid eliminating some labile enzyme system ; for example the conversion of guinea pig serum to euglobulin destroys the acid labile component of guinea pig plasminogen. Moreover, it is reasonable to work with biological material that is as intact as possible in order to ascertain what to search for in a purified product. It was realized, however, that plasmin is not the only enzyme capable of splitting TAMe. Sherry and Troll (1954) showed that TAMe is hydrolyzed by thrombin ; Lepow, Ratnoff and Pillemer (1956) and Becker (1956) demonstrated that the first component of complement, C'1, is a TAMe esterase ; and recently Becker (1959) presented evidence that the permeability factor, PF/Dil, described by Miles and Wilhelm (1955) is also a TAMe esterase. Hence before considering the effect of the antigen-antibody reaction of TAMe esterases, it was necessary to define and characterize the TAMe esterase activity present in guinea pig serum.

MATERIALS AND METHODS

All experiments were performed with fresh, once-frozen, pooled guinea pig serum obtained by heart puncture 2–6 hr. before the experiment. The serum was allowed to clot in the cold, frozen immediately after separation, and thawed just before use. The guinea pig colony from which the serum pool was obtained was completely changed several times during the 2½ years of this investigation ; the number of animals contributing to a pool on any one day varied from 6–18 depending on the experimental need. No attempt was made to control variation due to seasonal factors or biological cycles. This sometimes led to large variations in the absolute amounts of enzyme activity from one experiment to another. However, conclusions were drawn only from comparisons made within a given experiment. In addition, each experiment was repeated with different pools at different times until it was certain that the phenomenon observed was not peculiar to a single pool or a single time. Chloroform treated serum was prepared by adding 0·4 ml. chloroform (U.S.P.) per ml. serum, shaking vigorously in a thick-walled glass centrifuge tube for 4 min., centrifuging in the cold for 30 min. at 10,000 r.p.m., and immediately removing the supernatant serum. Further removal of the chloroform from the supernatant by evaporation was not found to be necessary. Peptone treated serum was obtained by the addition of 10 per cent. Difco peptone solution to give a final concentration of 50–100 mg. per ml. of serum.

Human euglobulin (containing " proactivator ") was prepared before each experiment from a pool of frozen human serum as follows : 1 ml. serum was diluted with 19 ml. distilled water in the cold, brought to pH 5·2 with cold 2 per cent acetic acid, allowed to stand 10 min. in the cold, and centrifuged 10 min. in the cold at 2000 r.p.m.; the precipitate was dissolved in 3·0 ml. of 0·15 M saline to give 1:3 human euglobulin. Streptokinase (Varidase Lederle), 100,000 unit, was dissolved in 5 ml. distilled water before each experiment.

The substrates p-toluene sulfonyl L-arginine methyl ester (TAMe), N-acetyl L tyrosine ethyl ester (ATEe), and L-lysine ethyl ester (LEe) or methyl ester (LMe) were obtained commercially (Mann Research Laboratories, Inc). Soybean trypsin inhibitor (SBI ; Worthington Biochemical Corp) was dissolved in 4 parts 0·15 M saline and 1 part 0·5 M tris (hydroxymethyl) aminomethane (tris) buffer pH 7·5.

The esterase activity of serum was measured by a slight modification of the formol titration method of Troll, *et al.* (1954) when TAMe or ATEe was used as a substrate. Serum was added to substrate, saline, and the appropriate buffer to give a final volume of 4·0 ml., and 1·0 ml. immediately removed for the zero time determination. The remaining 3·0 ml. were incubated at 37°, and 1·0 ml. was removed at 25 and 45 min. In order to maintain linear activity with time samples were taken at 10 and 20 min. or 15 and 30 min. when the serum preparation was particularly active. Each sample was added to 1·0 ml. of 37 per cent formaldehyde, pH 7·0–7·5. The amount of acid produced was then determined by measuring the amount of 0·06 N NaOH required to bring the reaction mixture to a pH of 7·2. The NaOH added

was measured by a Scholander (1942) microburette, and the pH was determined by means of a Beckman Probe Electrode attached to a Beckman Model G pH meter (Iselin and Niemann, 1950). A mechanical stirring device was used to obtain adequate mixing of the added NaOH. Serum activated with human euglobulin-streptokinase (Eu-SK) was incubated for 5 min. at 37° before mixing with the substrate. When the effect of SBI was studied the Eu-SK activated serum was incubated with the inhibitor for 2 min. before the addition of substrate.

In general, when plasmin was studied, the reaction mixture contained 0·8 ml. of 0·1 M substrate, 0·4 ml. of serum, 2·0 ml. of 0·5 M tris buffer, 0·3 ml. of 1 : 3 human euglobulin, 0·2 ml. SK (10,000 units/ml.), and saline to bring the volume to 4·0 ml. By prior test it was found that these proportions of streptokinase and human euglobulin gave maximal activation. When unactivated serum was studied 0·8 ml. of serum was used. Since changing the concentrations of tris from 0·1 to 0·5 M did not alter activity, it was found convenient to use 0·5 M buffer when a final pH greater than 8·5 was desired and 0·1 M buffer when a lower final pH was indicated. However, in all instances where the pH activity curves were determined 0·5 M buffer was used. Activity in all experiments has been expressed as micromoles of acid produced per 30 min. per ml. of guinea pig serum, and has been corrected by subtracting the spontaneous breakdown of substrate and the activity contained in the activating materials.

When lysine ethyl or methyl ester was used as substrate, the esterase activity was measured by chelation of the liberated lysine with copper. The method was essentially that described by Hagan, Ablondi, and Hutchings (1956) based on the observations of Spies and Chambers (1951). Two ml. of reaction mixture was prepared from guinea pig serum, phosphate buffer, substrate, streptokinase, human euglobulin and saline. After incubation for 1 hr. at 37°, 2 ml. of copper phosphate suspension was added to each reaction mixture giving a final volume of 4·0 ml. These tubes were then mechanically shaken for 5 min. and centrifuged for 15 min. at 3000 r.p.m. The supernatant was decanted into a colorimeter tube and read in a Junior Coleman spectrophotometer at 620 mμ. Yield was expressed in μM lysine liberated per 30 min. per ml. of guinea pig serum by comparing the colorimeter reading with a standard lysine curve. Before comparison, the reading was corrected by subtracting the spontaneous breakdown of substrate, the activity contained in the activating materials, and the optical density of the serum alone.

A 0·2 M phosphate buffer giving a final concentration in the reaction mixture of 0·075 M was satisfactory in the pH range of 5·5 to 7·5. However, in order to obtain a final pH of the reaction mixture above 7·5, it was necessary to add small quantities of 5 N NaOH.

In general, when plasmin was studied, the reaction mixture contained 0·5 ml. guinea pig serum, 0·25 ml. of 0·336 M LEe or LMe, 0·75 ml. of 0·2 M phosphate buffer, 0·15 ml. of 1:3 Eu, 0·1 ml. of SK (20,000/ml.), and saline to bring the mixture to a final volume of 2·0 ml. The activity of plain and chloroform-treated serum was so low that it was necessary to use 1·0 ml. of serum when these preparations were studied.

RESULTS

The guinea-pig TAMe esterase activity induced by Eu-SK

In Figure 1 the pH activity curve of untreated guinea pig serum TAMe esterase is compared with the pH curve of the same serum activated with Eu-SK to form plasmin. Whereas the pH optimum of the TAMe esterase of untreated serum was around 8·0, the pH optimum of guinea pig plasmin was around 9·0. All studies of guinea pig plasmin were accordingly carried out at pH 9·0.

Table I compares the Eu-SK activation of plain serum, serum pretreated with chloroform, and serum pretreated with peptone. In the first experiment, Eu-SK activation of plain serum increased total activity from 31 to 61 μM of acid per 30 min. per ml. of guinea pig serum. The increment of 30 μM represents the activity resulting from the conversion of plasminogen to plasmin. Chloroform treatment of the same serum increased activity by 19 μM, and Eu-SK activation of the chloroform-treated serum gave a further increment of 105 μM of acid per 30 min. per ml. of serum. In the second experiment, Eu-SK activation of plain serum

increased total activity from 25 to 39 μM of acid per 30 min. per ml. of serum. Peptone treatment of the same serum increased activity by 6 μM, and Eu-SK activation of the peptone-treated serum gave a further increment of 39 μM of acid per 30 min. per ml. of serum, representing the conversion of plasminogen to plasmin. Thus, the plasmin activity obtained by the Eu-SK activation of serum pretreated with chloroform or peptone significantly exceeded the activity

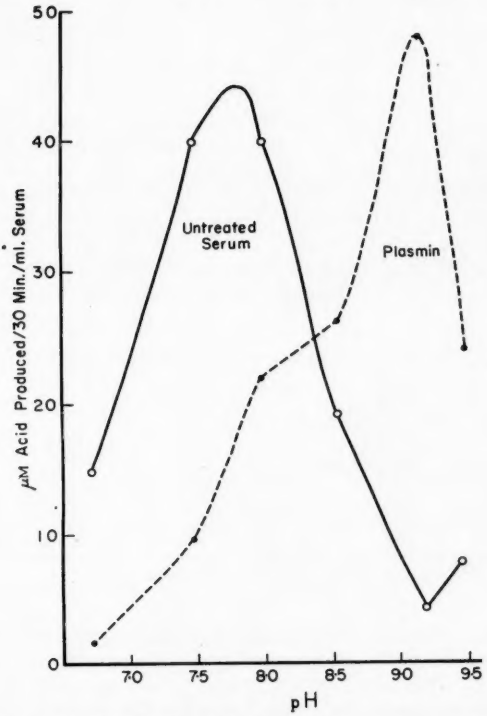

FIG. 1.—TAMe esterase activity of untreated guinea-pig serum compared with activity due to Eu-SK activation to yield plasmin.

obtained by activation of plain serum. The pH activity curves of plasmin of plain serum, chloroform-treated serum, and peptone-treated serum were superimposable with an optimum around pH 9·0. None of the Eu-SK activated sera was capable of splitting ATEe.

Table II compares the effect of SBI on the activity of plasmin obtained by the Eu-SK activation of plain serum, chloroform-treated serum, and peptone-treated serum. In the first experiment, SBI decreased the plasmin activity of plain serum from 39 to 16 μM of acid per 30 min. per ml. of serum, a reduction of 59 per cent, while the plasmin activity of chloroform-treated serum was reduced 100 per cent, from 105 to 0 μM acid per 30 min. per ml. of serum. In the second experiment the plasmin activity of plain serum was again partially inhibited

TABLE I. *Activation of Guinea Pig TAMe Esterase*

Experiment	Method of activation	μM acid produced per 30 min. per ml. of serum: Total activity	Increment due to activation*
1	Untreated	31	—
	Eu-SK	61	30
	Chloroform	50	19
	Chloroform-Eu-SK	155	105
2	Untreated	25	—
	Eu-SK	39	14
	Peptone	31	6
	Peptone-EuSK	70	39

* Net activity due to last step in activation procedure (see text), *e.g.*, chloroform refers to increment in activity obtained by addition of chloroform to plain serum, and chloroform-Eu-SK refers to increment in activity obtained by the addition of Eu-SK to serum pretreated with chloroform.

TABLE II.—*The Effect of Soybean Trypsin Inhibitor on Guinea Pig TAMe Esterase at pH 9·0*

Experiment	Method of activation	Increment in μM acid produced per 30 min. per ml. serum due to activation*: No SBI	SBI added†	Per cent inhibition
1	Eu-SK	39	16	59
	Chloroform	19	11	42
	Chloroform-Eu-SK	105	−2	100
2	Eu-SK	14	3	79
	Peptone	6	7	0
	Peptone-Eu-SK	39	46	0

* See footnote, Table I.
† 500 μg. SBI per ml. added after completion of activation process.

by SBI (79 per cent), whereas the plasmin activity of peptone-treated serum was completely resistant to SBI. Moreover, further experiments showed that the plasmin activity of chloroform-treated serum was completely inhibited by a concentration of 100 μg. per ml. of serum, whereas the plasmin activity of peptone-treated serum was totally resistant to SBI even at a concentration of 500 μg. per ml. of serum. That the peptone itself did not interfere with the action of SBI was demonstrated by the addition of peptone to chloroform-treated serum before Eu-SK activation, Table III. The subsequent addition of SBI was almost completely effective in inhibiting the plasmin of chloroform-treated serum despite the presence of peptone.

The effect of heating plain serum at 56° for 30 min. on subsequent activation is shown in Table IV. In the first experiment, heating plain serum prior to Eu-SK activation reduced the plasmin activity from 47 to 27 μM acid per 30 min. per ml. of serum, a 43 per cent reduction. In contrast, heating plain serum before chloroform-treatment and Eu-SK activation did not diminish plasmin activity. In the second experiment, heating plain serum before Eu-SK activation again produced a partial loss of activity and heating before peptone treatment and Eu-SK activation resulted in an even greater loss of proenzyme. This suggested

TABLE III.—*The Effect of Soybean Trypsin Inhibitor on Guinea Pig TAMe Esterase at pH 9·0*

Method of activation	Increment in μM acid produced per 30 min. per ml. serum due to activation*		Per cent inhibition
	No SBI	SBI added†	
Eu-SK	18	22	0
Chloroform	48	21	56
Chloroform-Eu-SK	107	9	92
Chloroform-peptone	26	21	19
Chloroform-peptone-Eu-SK‡	119	2	99

* See footnote, Table I.
† SBI added after completion of activation process (500 μg. SBI/ml. serum).
‡ Peptone added to chloroform-treated serum before Eu-SK activation.

that the plasmin proenzyme of peptone-treated sera was heat labile, in contrast to the heat stable proenzyme of chloroform-treated serum. Further, it was found that when serum was held at pH 2·3 for 1 hr. at 0°. and then neutralized, the plasmin proenzyme of peptone-treated serum was destroyed whereas that of chloroform-treated sera was completely retained.

TABLE IV.—*Effect of Heating Serum Prior to Activation of Guinea Pig TAMe Esterase*

Experiment	Method of activation	μM acid produced per 30 min. per ml. serum				Per cent reduction in activation
		Unheated serum		Heated serum*		
		Total activity	Increment due to activation†	Total activity	Increment due to activation†	
1	Untreated serum	19	—	12	—	—
	Eu-SK	66	47	39	27	43
	Chloroform	28	9	29	17	0
	Chloroform-Eu-SK	96	68	113	84	0
2	Untreated serum	18	—	5	—	—
	Eu-SK	58	40	19	14	65
	Peptone	32	14	15	10	29
	Peptone-Eu-SK	91	59	23	8	87

* Serum incubated at 56° for 30 min. before activation.
† See footnote, Table I.

The LMe esterase activity induced by Eu-SK

In Fig. 2 the pH activity curve of the LMe esterase of plain serum is compared with the pH activity curves obtained by Eu-SK activation, chloroform treatment, and Eu-SK activation of chloroform-treated serum. The spontaneous activity was small, and the pH optimum of this activity was not significantly different from that of the plasmin of plain or chloroform-treated serum. It was not possible to determine whether or not the proenzyme in peptone treated serum gave rise to a LMe esterase because the lysine in the peptone invalidated the assay.

Fig. 3 presents the effect of TAMe on the LMe esterase activity of plasmin from plain and chloroform-treated serum. In both instances competitive inhibition

was demonstrated when the results were plotted according to Lineweaver and Burk (1934).

Table V compares the effect of SBI on the LMe esterase activity obtained by Eu-SK activation of untreated and chloroform-treated serum. The plasmin activity of plain serum was not significantly reduced by SBI, whereas the plasmin activity of chloroform-treated serum was reduced 81 per cent.

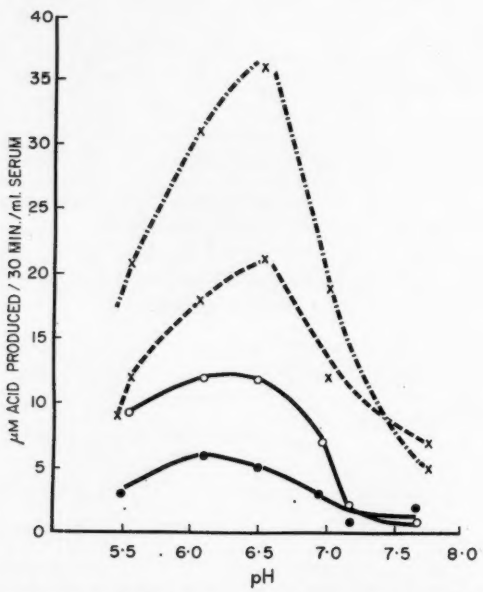

FIG. 2.—LMe esterase activity of untreated guinea-pig serum compared with activity after Eu-SK activation, chloroform activation, and Eu-SK activation of serum pretreated with chloroform. The activity plotted is the net activity due to the last step in the activation procedure (footnote Table I).

●——● Untreated serum.
×— —× Eu-SK-treated serum (Plasmin).
○——○ Chloroform-treated serum.
×—·—· × Chloroform-Eu-SK-treated serum (plasmin).

TABLE V.—*The Effect of Soybean Trypsin Inhibitor on Guinea Pig LMe Esterase at pH 6·6*

Method of activation	Increment in μM Lysine produced per 30 min. per ml. serum due to activation*		Per cent inhibition
	No SBI	SBI added†	
Eu-SK	14	13	7
Chloroform-Eu-SK	31	6	81

* See footnote, Table I.
† SBI added after completion of activation procedure (500 μg. SBI per ml. serum).

The effect of heating plain serum at 56° for 30 min. on subsequent activation is shown in Table VI. Heating plain serum before Eu-SK activation or before chloroform treatment and Eu-SK activation did not significantly diminish plasmin activity, indicating that both esterases arose from a predominantly heat stable proenzyme.

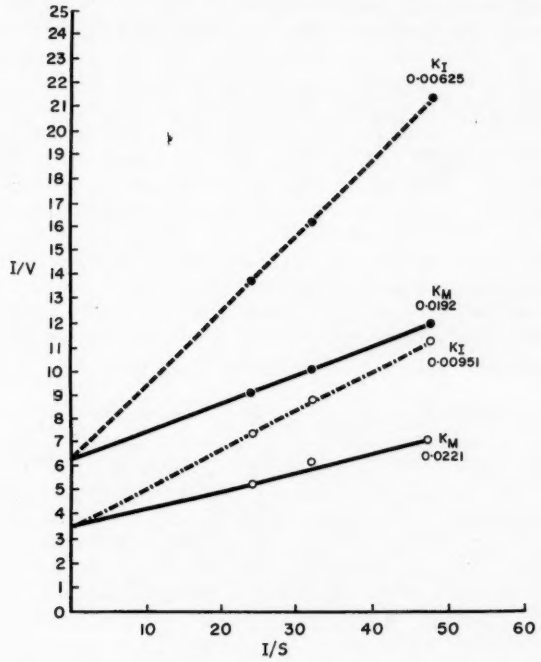

FIG. 3.—The effect of TAMe on the LMe esterase activity of guinea-pig serum activated by Eu-SK or by Eu-SK after pretreatment with chloroform. The activity plotted is the increment in activity due to Eu-SK activation to yield plasmin.

●— —● Eu-SK-treated serum plus TAMe.
●———● Eu-SK-treated serum.
○— —○ Chloroform-Eu-SK-treated serum plus TAMe.
○———○ Chloroform-Eu-SK-treated serum.

TABLE VI.—*Effect of Heating Serum Prior to Activation of Guinea Pig LMe Esterase*

Method of Activation	Increment in μM lysine produced per 30 min. per ml. serum due to activation*		Per cent reduction in activation
	Unheated serum	Heated serum†	
Eu-SK	16	14	13
Chloroform-Eu-SK	31	30	3

* See footnote, Table I.
† Serum incubated at 56° for 30 min. before activation.

Activation of guinea pig euglobulin by human euglobulin and streptokinase (Eu-SK) yielded a TAMe esterase, which, like the plasmin of chloroform-treated serum, was completely inhibited by SBI—even in the presence of peptone. This suggested that the heat and acid labile plasmin proenzyme of peptone-treated serum was lost when guinea pig euglobulin was prepared, whereas the heat stable, acid resistant, plasmin proenzyme of chloroform-treated serum survived euglobulin preparation. The possibility that the heat- and acid-labile proenzyme was not destroyed, but rather present in the supernatant was studied by preparing euglobulin by acidification and dialysis (Guinea pig serum was brought to pH 5·6 by 0·03M HCl and dialyzed overnight against a pH 5·4 phosphate buffer ($\mu = 0{\cdot}02$); after centrifugation the supernatant was removed and brought to pH 7·4 by 1·0 N NaOH; the precipitate was washed with pH 5·4 phosphate buffer ($\mu = 0{\cdot}02$) and dissolved in pH 7·6 phosphate ($\mu = 0{\cdot}02$)-saline (0·15M)). However, neither the supernatant nor the precipitate contained the labile proenzyme, as measured by the addition of peptone prior to Eu-SK activation. Furthermore, the LMe esterase activity resulting from the Eu-SK activation of guinea pig euglobulin was also completely inhibited by SBI, whereas similar activation of the whole serum invariably yielded an esterase that was predominantly SBI resistant. Thus, as measured by either substrate, the preparation of guinea pig euglobulin removed the SBI resistant proesterase.

The effect of antigen-antibody complexes on the Eu-SK induced TAMe esterase activity

Plain serum and serum treated with a rabbit antigen-antibody precipitate at 4° were compared with respect to TAMe esterase activity at pH 9·0. There was no change in the spontaneous activity. Furthermore, as shown in Table VII, the amount of plasminogen activated in plain serum, chloroform-treated serum, or peptone-treated serum was the same whether or not the serum had been pre-treated with the antigen-antibody precipitate. Other experiments, not shown herein, in which the serum was incubated with the precipitate at 37° for 1 hr. instead of at 4° also failed to demonstrate any effect of the antigen-antibody precipitate.

TABLE VII.—*The Effect of Treating Serum with an Antigen-Antibody Precipitate Prior to Activation of Guinea Pig TAMe Esterase*

Method of activation	Increment in μM acid produced per 30 min. per ml. serum due to activation*	
	Untreated serum	Serum treated with antigen-antibody precipitate†
Eu-SK	31	32
Chloroform-Eu-SK	104	102
Peptone-Eu-SK	67	61

* See footnote, Table I.

† Serum incubated with ovalbumin-rabbit antiovalbumin precipitate (total nitrogen 0·5 mg. per ml. guinea-pig serum) for 1 hr. at 4°.

The effect of the addition of an antigen, ovalbumin, on the esterase activity of hyperimmune antiovalbumin guinea pig serum was also investigated. There was no increase in the guinea pig TAMe esterase activity when the antigen was

added in equivalence or excess, and there was no apparent change in the concentration of plasmin proenzyme of plain serum, chloroform-treated serum, or peptone-treated serum. Because of these negative results, the activity was measured in the euglobulin precipitate of hyperimmune serum after addition of antigen excess, as suggested by Ungar and his co-workers (1949, 1953). As is shown in Table VIII, the addition of specific antigen in excess or equivalence failed to increase the TAMe esterase activity of the euglobulin preparation.

TABLE VIII.—*The Effect of the* In Vitro *Immune Reaction on the TAMe Esterase Activity of Guinea Pig Euglobulin and Whole Serum*

Preparation studied	μM acid produced per 30 min. per ml. antiserum*		
	No antigen	Equivalence	Antigen excess†
Whole antiserum . .	22	—	20
Euglobulin . . .	11	12	12

* Guinea pigs were immunized with egg albumin in Freund's adjuvant. One ml. antiserum (antibody concentration approximately 0·2 mg. antibody nitrogen per ml.) was treated with either saline or antigen. Euglobulin was prepared in the usual fashion by dilution and acidification, and the precipitate was dissolved in 1·0 ml. saline (1 : 1 euglobulin).

† Antigen excess in these experiments was 20 times equivalence.

DISCUSSION

These studies indicate that the plasminogen of plain guinea pig serum, which is partially and variably heat-labile and gives rise to a TAMe esterase which is partially and variably inhibited by SBI, consists of two or more plasminogens. The plasminogen of chloroform-treated serum, hereafter called plasminogen C, is heat stable and gives rise to a plasmin measured as a TAMe esterase, which is completely inhibited by the soybean trypsin inhibitor. In contrast, the plasminogen of peptone-treated serum, hereafter called plasminogen P, is heat labile and gives rise to a TAMe esterase that is completely resistant to the soybean trypsin inhibitor.

The LMe esterase activity arising fron the addition of Eu-SK to chloroform-treated serum is markedly inhibited by SBI, is competitively inhibited by TAMe, and arises from a heat stable precursor. This suggests that plasmin C is both a LMe and a TAMe esterase. On the other hand, since the LMe esterase activity of plain serum activated by Eu-SK arose from a predominantly heat stable precursor (87–100 per cent stable) and was always SBI resistant, it cannot be readily attributed to activation of either plasminogen P or C—the former being heat-labile and the latter giving rise to a plasmin that is inhibited by SBI. Indeed, these studies could be interpreted to suggest that there is another plasminogen that is heat stable and gives rise to a LMe esterase that is SBI resistant.

The suggestion that there may be more than one plasminogen has been made previously. Because bovine fibrinogen was lysed by tissue activator but not by streptokinase, Astrup and Permin (1948) postulated two different plasminogens. Subsequent work, however, suggested (Mullertz, 1953; Sherry, 1954) that the difference was due to the fact that streptokinase does not act directly on plasminogen but requires the presence of a "proactivator" lacking in bovine serum. Paly and Kline (1954), using dog plasma, presented evidence that the spontaneous

enzyme, which they called plasmin B, was different from the enzyme, plasmin, activated by staphylokinase. Although these differences exist, these studies can not be accepted as evidence for two plasminogens if we define plasminogen as a proenzyme activated by streptokinase or streptokinase plus human " pro-activator ", depending on the species of origin.

Troll and Sherry (1955) suggested that the human activator arises from a proenzyme, the " proactivator ", which though similar to plasminogen differs in several important respects. In contrast, Ablondi and Hagan (1957) proposed that human plasminogen and the human " proactivator " of bovine plasminogen are the same. It should also be noted that the results of Schultz and von Kaulla (1958) suggest that an enzyme analogous to plasminogen P may exist in human serum, although this was not the interpretation given by the authors. Definite conclusions regarding these points must await a more adequate characterization of human " proactivator ", the urokinase activated proenzyme, and plasminogen P.

We were unable to demonstrate that any of the plasminogens identifiable in our system were activated by an antigen-antibody reaction. This in no way invalidates the concept that the antigen-antibody reaction induces proteolytic activity which in turn is responsible for cell damage or the release of toxic substances. Our findings are merely additional evidence that it is not the plasmin system which is responsible. Both Burdon (1958) and Becker (1957) have recently reviewed the evidence that protease activity plays a role in histamine release and anaphylaxis and have pointed out that there is little evidence that plasmin is involved. The fibrinolytic blood protease present in a variety of disease states (Ratnoff, 1949 ; Clifton, 1952 ; Scott, Matthews, Butterworth and Frommeyer, 1954 ; Meyers, Burdon and Riley, 1957) is not associated with anaphylactic signs or symptoms. The intravenous administration of streptokinase (Tillett, Johnson and McCarty, 1955) or the intrathecal administration of plasmin (Fletcher, 1954) to human subjects is not characterized by any of the signs or symptoms of clinical allergy. Moreover, SK-activated fibrinolysin does not cause histamine release from rabbit red blood cells (McIntire, 1956) or platelets (Humphrey and Jaques, 1955) or from isolated perfused guinea-pig lung (Roche e Silva, 1956). Hence, the failure of the antigen-antibody reaction to influence guinea pig plasminogen was not unexpected. On the other hand, there is an enzyme system in guinea pig serum which is affected by the antigen-antibody reaction (Becker, Austen and Marcus, 1959).

SUMMARY

The plasminogen of guinea pig serum is a mixture of at least two proenzymes, plasminogen C and plasminogen P. Plasminogen C is unaffected by heating at 56° for 30 min. and gives rise to a p-toluene sulfonyl L-arginine methyl ester esterase and a L-lysine methyl ester esterase that is completely inhibited by soybean trypsin inhibitor. Plasminogen P is heat labile, and gives rise to a p-toluene sulfonyl L-arginine methyl ester esterase that is resistant to soybean trypsin inhibitor. Moreover, plasminogen P is destroyed when euglobulin is prepared from guinea pig serum, whereas plasminogen C survives euglobulin preparation. Neither plasminogen C nor plasminogen P is affected by a rabbit antigen-antibody precipitate, nor when ovalbumin is added to hyperimmune antiovalbumin guinea pig serum.

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TAMe ESTERASE ACTIVITY OF GUINEA-PIG SERUM

II. PEPTONE INDUCIBLE ESTERASES

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THE injection of peptone into animals produces a type of shock which in many particulars simulates anaphylaxis (Biedl and Kraus, 1909; Roche e Silva, 1955). This has led to the speculation that the underlying mechanisms in peptone and anaphylactic shock are very similar if not identical. Moreover, the addition of peptone to serum induces proteolytic activity in the serum (Ungar, Damgaard and Hummel, 1953; Olesen, 1957), which in turn has been cited as supporting evidence for the theory that the induction of proteolytic activity is the fundamental mechanism of anaphylactic shock.

During the study of guinea-pig plasmin (Austen, Becker and Marcus, 1959) it was observed that the addition of peptone to guinea-pig serum increased the *p*-toluene sulfonyl L arginine methyl ester (TAMe) esterase activity measured at both pH 8 and pH 9. We show in this paper that the increase in activity measurable at the two pH values is due to the activation by peptone of two distinct enzymes, and discuss the findings in relation to the hypothesis that peptone shock is due to the activation of a proteolytic enzyme.

The effect of the antigen-antibody reaction on the two peptone inducible esterases is described. In this connection Lepow and his colleagues (Lepow, Ratnoff and Pillemar, 1956; Lepow, Ratnoff and Levy, 1957; Ratnoff and Lepow, 1957) working with human serum, and Becker (1956*a*, *b*; 1959*a*) with guinea-pig serum, showed that the first component of complement (C'1) is an esterase capable of splitting TAMe and acetyl L tyrosine ethyl ester (ATEe) as well as certain other amino acid esters. They both concluded that the enzyme exists in serum as a proenzyme which is activated during the course of the antigen-antibody reaction. Lepow *et al.* (1956), eluted such an esterase from antigen-antibody precipitates treated with fresh human serum. Becker (1959*a*) eluted a similar esterase, and material with C'1 activity from sensitized sheep erythrocytes treated with fresh guinea-pig serum. The properties of the enzymes induced by the addition of peptone were therefore compared with those of the enzyme eluted from sensitized sheep red cells treated with guinea-pig serum. Certain striking similarities as well as differences were noted.

MATERIALS AND METHODS

The fresh guinea pig serum, peptone, buffer, substrates and esterase assay were as described by Austen, Becker and Marcus (1959). Ethyl cellosolve in a final concentration in the assay mixture of 10 per cent was used to dissolve the acetyl L tryrosine ethyl ester (ATEe).

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The anti-egg albumin antiserum containing 0·65 mg. antibody N/ml., was a pool obtained by the subcutaneous injection into rabbits of crystalline egg albumin emulsified in Freund's adjuvant (Freund and Bonanto, 1944). The rabbit antipneumococcal type I antiserum and the type I pneumococcal polysaccharide were obtained through the generosity of Mr. Arthur Abrams. The antiserum contained 0·2 mg. antibody N/ml. Dr. Irwin Lepow generously provided the rabbit antipneumococcal type III (4·0 mg. antibody N/ml.) as well as the type III pneumococcal polysaccharides.

For all experiments antigen-antibody precipitates were formed at equivalence. They were washed twice in the cold with a saline (0·15 M) phosphate (0·02 M) buffer, pH 7·6. Ice cold guinea pig serum was added to the precipitate in the proportion of 0·2 or 0·3 mg. AbN per ml. of serum. The precipitate, suspended as finely as possible, was allowed to stand in the cold for 30 min. with occasional inversion of the tube. The mixture was then centrifuged at 10,000 r.p.m. for 20 min. and the supernatant poured off and tested for esterase activity. In those experiments in which the esterase content of the precipitate was also to be determined the precipitates were washed twice with 0·04 M NaCl as described by Lepow (personal communication), suspended in a finely divided form in 0·15 M NaCl and analyzed for esterase activity in the usual way.

The rabbit haemolysin (SH-1 ; Becker, 1959*a*) used to sensitize the sheep erythrocytes was a pool of high-titred serum obtained from commercial sources. The sheep red cells were sensitized with a 1–100 dilution of this haemolysin, a concentration found (Becker, 1959*a*) to give nearly maximal uptake of esterase. The haemolytic activity of the guinea pig serum was destroyed by the addition of sufficient formaldehyde to inactivate the third component (C'3) of complement. Formaldehyde-inactivated serum was used in preference to fresh serum in order to lessen the haemolysis that would otherwise result, even in the cold, if undiluted, untreated serum was mixed with the packed, highly-sensitized cells. For this purpose, 1 ml. of serum was treated with 0·17 ml. of 0·185 M formaldehyde at 37° for 30 min. (Rapp and Sims, 1958). This treatment was shown by prior test not to affect the esterases under investigation.

In experiments on the effect of sensitized erythrocytes on the peptone induced activity of guinea pig serum the cells were centrifuged at high speed in the cold and then the ice cold formaldehyde treated serum was added in the proportion of 0·5 ml. of serum to 10^{10} cells. The serum and cells were thoroughly but gently mixed and allowed to stand in the cold for 20 min. The mixture was centrifuged and the esterase activity of the supernatant compared with the esterase activity of the control formaldehyde treated serum. The cells were discarded except in those experiments in which the esterase activity picked up by the cells was to be determined. In such experiments the cells were washed in buffered saline containing Mg^{++} and Ca^{++} and the TAMe esterase activity of 5×10^9 cells was measured by the Hestrin (1949) assay after 4 hr. at 37° (Becker, 1959*a*).

RESULTS

The activation of guinea-pig serum esterase measured at pH 8·0 and pH 9·0

As already demonstrated (Austen *et al.*, 1959) the addition of peptone increases TAMe esterase activity when measured at pH 9·0 ; however, as shown in Table I, an even greater increase was found when the activity was determined at pH 8·0.

Table IIA shows that merely allowing the serum to stand at 37° for an hour or more increases the esterase activity measured at pH 8·0. Moreover, this increase in activity is equal to the increase occurring after the addition of peptone to fresh serum while no further increase results from the addition of peptone to incubated serum. On the other hand, when the activities were measured at pH 9 (Table IIB) there was little or no increase in activity following incubation at 37° and no change in the ability of peptone to cause activation after such incubation. In this experiment TAMe was used as the substrate for the determinations carried out at pH 8·0, while ATEe was used as the substrate for the determinations carried out at pH 9·0. ATEe was chosen in this experiment

because, as will be shown later, the enzyme measured at 9·0 responds more actively to this substrate than to TAMe. In other experiments at pH 9 identical results were obtained using TAMe throughout.

Table III shows that 5-day incubation at 4° also results in an increase in activity when measured at pH 8·0, but not when measured at pH 9·0.

TABLE I.—*The Effect of Peptone Addition on TAMe Esterase Activity Measured at pH 8·0 and pH 9·0*

pH of measurement	μM of acid produced/ml. of serum/30 min.		
	Without peptone	With peptone	Peptone increment (Δ peptone)
8·0	30	50	22
9·0	19	32	13

TABLE II.—*The Effect of Incubation of Serum at 37° on the Peptone-Induced Esterase Activity Measured at pH 8·0 and pH 9·0*

	Treatment	μM acid produced/ml. of serum/30 min.	
		No prior incubation	80 min. incubation at 37°
A. Activity measured at pH 8·0 (0·020M TAMe)	Without peptone	30	42
	With peptone	41	41
	Δ peptone	11	0
B. Activity measured at pH 9·0 (0·20M ATEe)	Without peptone	15	12
	With peptone	24	20
	Δ peptone	9	8

TABLE III.—*The Effect of Incubation of Serum at 4° on the Esterase Activity Measured at pH 8·0 and pH 9·0*

	μM of acid produced/ml. of serum/30 min. TAMe esterase activity measured at	
	pH 8·0	pH 9·0
Fresh serum	36	33
Serum standing 5 days at 4°	53	29

The pH activity curve of TAMe esterase activated by the addition of peptone

The increase of activity obtained on treatment of unheated serum with peptone plotted against the pH (Fig. 1) yielded a bimodal curve with one peak around pH 8·0 and another around pH 9·0. Heating completely eliminated the peak at pH 8·0 but had no effect on the peak at pH 9·0.

The substrate specificity of the peptone inducible esterases

Previous work has demonstrated that the esterase identified with the first component of complement (C'1) is not only active against TAMe (Ratnoff and Lepow, 1957) but ATEe as well (Lepow, personal communication). This suggested testing the activity of heated and unheated guinea-pig serum against these two substrates at both pH 8·0 and pH 9·0. It is evident from Table IV that against

ATEe the activity at pH 8·0 was only 40 per cent of that found when TAMe was used as the substrate. At this pH, the activity against both substrates was reduced to essentially zero when the serum was heated to 56° for 30 min. At pH 9·0, however, the activity against ATEe was double that found with TAMe, and the activities against both substrates were unchanged after heating to 56°.

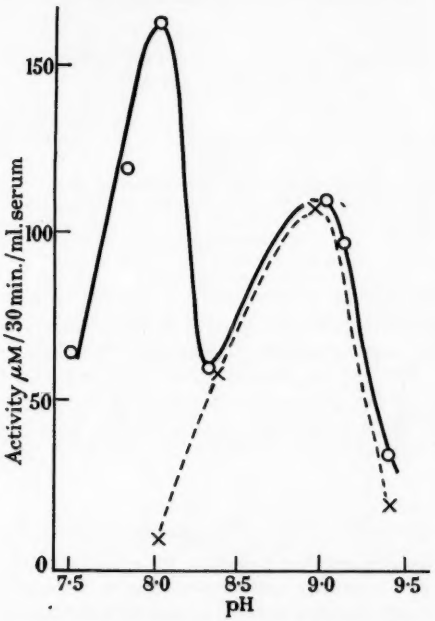

FIG. 1.—The pH activity curve of the peptone induced TAMe esterase activity of guinea-pig serum

○———○ Unheated serum.
×– – –× Heated serum (56° for 30 min.).

TABLE IV.—*The Effect of Substrate on the Peptone Induced Esterase Activity of Heated and Unheated Guinea-pig Serum at pH* 8·0 *and pH* 9·0

	Increment of activity due to peptone addition μM acid produced/ml. of serum/30 min.			
	pH 8·0		pH 9·0	
Serum Treatment	TAMe	ATEe	TAMe	ATEe
Unheated serum . .	11	4·4	2·7	5·4
Heated serum . .	0·9	0·9	2·7	6·0

The substrate specificity of the peptone-induced esterase with a pH optimum at 8·0 was more extensively investigated. From the results in Table VA, it is evident that TAMe, benzoyl L arginine methyl ester (BAMe) and ATEe are all active as substrates, the ratio of activities being 1·0 : 0·51 : 0·36, respectively.

This is essentially the same ratio of activities (1·0 : 0·41 : 0·36) previously recorded for the esterase isolated from sensitized sheep cells treated with guinea-pig serum (Becker, 1959*b*). Peptone induced no esterase activity when L leucine ethyl ester, L phenylalanine ethyl ester and DL serine ethyl ester were employed as substrates (Table VB). In this respect also it resembled the esterase isolated from sensitized sheep cells treated with guinea-pig serum.

TABLE V.—*The Effect of Substrate on Peptone Inducible Esterase Activity Measured at pH 8·0*

Experiment	Substrate	Peptone increment μM/ml. serum/30 min.
A	TAMe	15·5
	Benzoyl L arginine methyl ester	7·9
	ATEe	5·6
B	TAMe	11·5
	L-leucine ethyl ester	0
	L-phenylalanine ethyl ester	0
	DL-serine ethyl ester	0

The effect of peptone on activity against L lysine ethyl ester (LEe) could not be tested because the large amount of peptides and simple amino acids in the peptone reacted with the copper reagent used to assay the L lysine ethyl ester. Through the kindness of Dr. Robert Berg a sample of a purified peptide fraction, which he had isolated from peptone was obtained. This induced an increase in activity measured at pH 8·0 when TAMe was used as a substrate, but not when LEe was employed.

The effect of antigen-antibody reaction on peptone induced TAMe esterases

The addition of fresh guinea-pig serum to sensitized sheep red cells causes TAMe esterase activity to appear on the sensitized cells (Becker *et al.*, 1959*a*); and the addition of fresh human serum to antigen-antibody precipitates causes ATEe esterase to appear on the precipitate (Lepow, personal communication). These findings suggested that treatment of guinea-pig serum with an antigen-antibody complex would result in a decrease in the peptone-induced TAMe esterase of the serum.

In the first experiment (Table VI) two antigen-antibody systems were used, a pneumococcal-rabbit antipneumococcal precipitate formed at equivalence and sensitized sheep erythrocytes. The pneumococcal rabbit antipneumococcal system (Table VIA) removed almost 60 per cent of the peptone-activatable TAMe esterase measured at pH 8·0, but had no effect on the esterase measured at pH 9·0. With the red cell system the differences were even more striking (Table VIB). Here all the peptone activated esterase measured at pH 8·0 was removed whereas there was little if any decrease in that at pH 9·0.

These experiments showed that different antigen-antibody systems varied in their ability to remove peptone activable esterase. Other experiments revealed that the same antigen-antibody system would remove widely different amounts of proesterase from different guinea-pig serum pools. This is illustrated by the results of two experiments (Table VII). In both the egg albumin and the pool of rabbit anti-egg albumin were the same. Yet in Experiment A, almost 81 per cent of the peptone-inducible esterase was removed while in Experiment B

TABLE VI.—*Effect of Antigen-Antibody Complexes on Peptone-Induced Esterases*

	μM acid produced/ml. of serum/30 min.					
	pH 8·0			pH 9·0		
	No peptone	Peptone	Δ peptone	No peptone	Peptone	Δ peptone
Experiment A—						
Untreated serum . .	26	42	16	13	24	11
Serum treated with immune precipitate*	31	38	7	12	25	13
Experiment B†—						
Untreated serum . .	28	35	7	4	13	9
Serum treated with sensitized sheep red cells‡	24	24	0	5	12	7

* 0·3 mg. of antipneumococcal polysaccharide type I AbN in the precipitate/1 ml. of serum.
† 0·02M TAMe used when measurements were carried out at pH 8 and 0·02M ATEe used when the activity was measured at pH 9.
‡ 0·5 ml. of serum was treated with 10^{10} cells.

there was a loss of only 14 per cent. The reason for the difference is not known. It is of interest, however, that in both experiments there was a complete loss of the first component of complement activity (C'1).

TABLE VII.—*Comparison of the Results of Two Different Experiments on the Effect of Egg Albumin-Rabbit Anti Egg Albumin Precipitate on the Peptone-induced Esterase Activity*

	μM acid produced/ml. of serum/30 min.			
	Experiment A		Experiment B	
	Control	AgAb treated	Control	AgAb treated
Untreated	28	27	19	23
Treated with peptone . .	45	30	33	35
Δ peptone	17	3	14	12

Comparison of the Peptone Induced Esterase (Optimum at pH 8·0) Removed from Serum by Antigen-Antibody Complex with the Amount of Esterase Recovered in the Complex

Since a relatively large amount of peptone-induced esterase measurable at pH 8·0 was removed by certain antigen-antibody complexes attempts were made to see whether comparable amounts could be recovered from the complex. Lepow (personal communication) found relatively high amounts of ATEe esterase activity in type III pneumococcus polysaccharide-rabbit anti polysaccharide precipitates treated with human serum. Through the generosity of Dr. Lepow a sample of the same antiserum and type III polysaccharide was obtained. The recovery of TAMe esterase activity in precipitates treated with guinea-pig serum and human serum was then compared by the procedure outlined by Lepow and described above. It is evident from Table VIII that when the antibody precipitate is treated with guinea-pig serum the TAMe esterase activity found on the precipitate represents only a small percentage of the peptone-inducible activity which is lost from guinea-pig serum. However, when the same type and amount of precipitate was used to treat human serum the amount of esterase

found in the precipitate was almost 70 per cent of the peptone-inducible esterase of human serum.

TABLE VIII.—*The Uptake of TAMe Esterase by Pneumococcus Type III Polysaccharide-Rabbit Antipolysaccharide Precipitates When Treated With Either Human or Guinea-pig Serum*

	μM acid produced/ml. of serum (or equivalent of precipitate)/ 30 minutes	
	Guinea-pig serum	Human serum
Δ peptone of fresh serum	14·6	7·3
Δ peptone of antigen antibody treated serum	2·2	0
Activity of precipitate treated with serum	1·9	4·8
Per cent recovery of peptone activity in precipitate	13	67

The uptake of TAMe esterase by egg albumin-anti egg albumin precipitate from guinea-pig serum was only 5–10 per cent of the peptone induced esterase disappearing from the serum. Sensitized sheep red cells were treated with guinea-pig serum and the amount of esterase taken up by these cells was compared to the amount of peptone-inducible activity originally present in the serum (Table IX). In contradistinction to the results obtained with antigen-antibody precipitates the esterase activity taken up from guinea-pig serum by sensitized red cells was over 60 per cent of the peptone-inducible activity. This is the same range as that found when the polysaccharide antipolysaccharide precipitate was treated with human serum.

TABLE IX.—*The Uptake of TAMe Esterase by Sensitized Sheep Red Cells Treated with Guinea-pig Serum*

	μM of acid produced/ 30 min./ml. of serum
Δ peptone of formaldehyde inactivated guinea-pig serum	18
Δ peptone of formaldehyde inactivated guinea-pig serum after treatment with sensitized red cells	0
Esterase activity of sensitized cells treated with formaldehyde inactivated guinea-pig serum	11*
Esterase activity of sensitized cells not treated with serum	0

* μM of ester broken down/30 min./2 × 10^{10} sensitized cells treated with 1 ml. of serum.

DISCUSSION

It is evident that the effects of peptone on the TAMe esterase activity of guinea-pig serum are extremely diverse. Streptokinase and human activator are able to activate one of the plasminogens of guinea-pig serum, plasminogen P, to a much greater extent in the presence of peptone than in its absence (Austen *et al.*, 1959). In addition, peptone alone causes an increase in TAMe esterase activity whether measured at pH 8 or 9. Our evidence indicates that the increase is due to the activation by peptone of two separate and distinct enzymes. One enzyme arises from a heat labile precursor, has a pH optimum around 8 and has distinctly greater activity against TAMe than against ATEe. The pro-esterase is also activated by standing 1 hr or more at 37° or 3–5 days at 4°. The esterase

activity due to this enzyme disappears to a variable extent when the serum is treated with an antigen-antibody complex. The second enzyme arises from a heat-stable precursor, has a pH optimum around pH 9 and has distinctly greater activity against ATEe than against TAMe. It is not activated by standing either at 37° or at lower temperatures and is not affected by antigen-antibody complexes, under the conditions studied. Neither enzyme is inhibited by SBI.

Peptone, however, does more than affect esterases. The fibrinolytic activity induced by the addition of peptone to guinea-pig serum is apparently due to the activation of an enzyme which is not an esterase (Becker, 1959*b*) and so is apparently neither plasmin nor any of the other esterases previously discussed. Thus peptone has been shown to affect at least four different and distinct enzymes. The preparation used in this work and in most biochemical and physiological experiments is an extremely complex mixture of amino acids, peptides and larger protein fragments. It is therefore impossible to state whether the activation of the various enzymes is due to a single substance or whether different constituents activate different enzymes. However, with such a wealth of enzymes affected by peptone it seems premature to ascribe the pronounced *in vivo* effects of peptone to the activation of any one of them.

Lepow working with human serum and Becker with guinea-pig serum identified the first component of complement (C′1) as a TAMe esterase. The properties of the TAMe esterase activated by peptone with a pH optimum around 8 correspond to those of Becker's esterase from guinea-pig serum associated with C′1. However, as we have shown, the amount of pro-esterase removed by an antigen-antibody precipitate can vary from 0 to almost 100 per cent, under circumstances in which the C′1 activity is completely or almost completely removed. This may mean that the peptone-induced enzyme is not the esterase identified by both Lepow and Becker as C′1, or that our present ideas as to what happens in the fixation of C′1 will have to be enlarged.

The uncertainty about the identity of the peptone-induced esterase does not detract from the finding that antigen-antibody complexes are capable of removing it from serum. The effect of various antigen-antibody complexes on this esterase was, however, quite variable and presented a number of puzzling features. With the egg albumin-anti egg albumin precipitates the amount of enzyme which disappeared from guinea-pig serum varied from 0 to 100 per cent. But no matter what percentage disappeared from serum, no more than 5–10 per cent of enzyme activity could be found in the precipitate. The carbohydrate-anti carbohydrate precipitates behaved similarly. The sensitized red cell, on the other hand, regularly removed practically all of the esterase and at least 60 per cent of this activity could be found on the cells. Since one does not know the amount of antibody on the sheep red cells, it is at least possible that the difference between the sensitized red cell and the precipitate is only quantitative. It is more likely, however, that the amount of antibody on the red cell was probably less than that in the precipitates and that the explanation must be sought elsewhere. It is unlikely that the relative ineffectiveness of the precipitates to bind esterase is a general characteristic of antigen-antibody precipitates since, as shown by Lepow and confirmed by us, the pneumococcal polysaccharide-anti polysaccharide precipitate is capable of binding a large proportion of esterase from human serum under circumstances where it fails to bind the enzyme from guinea-pig serum. The effect of serum is also seen in the variation of the amount

of pro-esterase which disappears from different pools of guinea-pig serum treated with identical amounts of egg albumin-anti egg albumin precipitate prepared from the same antiserum under the same conditions.

SUMMARY

The addition of peptone to guinea-pig serum causes an increase in the activity of two *p*-toluene sulfonyl L arginine methyl ester (TAMe) esterases, one with a pH optimum around 8 and the other at 9. These enzymes also differ in their substrate specificity, the heat stability of the pro-esterase and the activatability of the pro-esterase on standing.

The addition of various antigen-antibody complexes to guinea-pig sera resulted in the virtual disappearance of the enzyme with the pH optimum around 8 from some sera ; but this effect was not consistent.

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CELLULAR CHANGES INDUCED BY CADMIUM IN MOUSE TESTIS AND LIVER

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VERY little is known of the biological role of zinc. It is widely distributed in body tissues, with comparatively high concentrations in spermatozoa (Mawson and Fischer, 1953). High levels have also been reported in the tissue of argentaffinomata (Feyrter, 1956), and Heath (1949) found a greater content of zinc in human rectal carcinoma than in the surrounding normal tissue. Abnormally low serum levels were encountered by Vallee, Wacher, Bartolomay and Robin (1956) in cases of cirrhosis of the liver.

Fujii (1954) reported the presence of zinc in nucleoli and its appearance in the chromosomes as soon as the nucleoli disappeared during mitosis. Heath (1949) demonstrated that deoxyribonucleoprotein contained natural zinc and also showed a considerable uptake of radioactive zinc. Furthermore, deoxyribonuclease (the enzyme which depolymerises deoxyribonucleic acid) is inhibited by zinc (Murray and Daly, 1958). In the light of these observations it seemed possible that some degree of disorganisation of mitosis would result from a disturbance of zinc metabolism.

From the physicochemical point of view cadmium is closely related to zinc and its administration might therefore interfere with the action of zinc to some extent. As testis contains comparatively large amounts of zinc (Mawson and Fischer, 1953) and also exhibits a high mitotic rate, this appeared to be the tissue in which any effects of cadmium on zinc metabolism might be most suitably observed.

Accordingly, a solution of a cadmium salt was injected into mice. There ensued destruction of testicular tissue. A search of the literature revealed an earlier similar observation by Parizek (1957) who counteracted this effect by the simultaneous administration of large quantities of zinc, and demonstrated that this action of cadmium was very probably due to a disturbance of zinc metabolism. Parizek surveyed the literature but was unable to find any previous report of this biological antagonism between zinc and cadmium in higher animals or of the destructive effect of cadmium on testis.

In the initial stage of the investigation, the optimal dose level was not known and some animals either showed no effect at all or else succumbed to acute toxicity. This paper describes the early pathological changes seen in testicular cells and occasionally in liver following the injection of a cadmium salt solution.

METHOD AND MATERIALS

Thirty male mice of an inbred albino strain, about three months old, received a single subcutaneous dorsal injection of a sterile solution of cadmium chloride (0·1 per cent in physiological saline) at a dosage level of 0·5 ml. per 100 g. body weight. (Others received

doses at levels ranging from 0·15 to 1·5 ml. per 100 g. but these results are not considered here). Male mice of similar age and strain used as controls received no treatment.

The animals were killed in pairs by excess anaesthesia at 2, 4, 6, 8, 10, 12, 16, 20, 24, 48, 72 and 96 hr. and also at 5, 21 and 49 days. The testes were removed immediately, bisected and placed in fixative, one from each animal in San Felice and the other in buffered neutral formalin. Paraffin-embedded sections cut at 3μ were used for staining with haematoxylin and eosin, periodic-acid-Schiff and Feulgen.

RESULTS

In general the animals were listless for about 24 hr. following the injection but subsequently behaved normally and appeared to be quite healthy.

At autopsy the following changes were seen. At about 12 hr. the testes appeared macroscopically to be swollen and dark red in colour. At 24 hr. these remained swollen but the colour had usually returned to normal. The swelling had subsided in most cases about 48 hr., and by the twenty-first day the testes were distinctly shrunken and yellow. Microscopically, the earliest change seen was oedema of the interstitial tissue, and vascular engorgement, which appeared by about 14 hr., was frequently gross in degree (Fig. 1), and often persisted until 48 hr.; interstitial haemorrhage was not uncommon, and thromboses were found occasionally (Fig. 3).

By 24 hr. karyolysis, karyorrhexis and pyknosis was seen in some of the cells of the seminiferous epithelium. The Sertoli cells were not affected at this stage, and there was no evidence of damage to the cells of the intertubular tissue.

This was followed soon by the appearance of numerous giant cells. These consisted of discrete clumps of granular eosinophilic cytoplasm containing up to 18 nuclei (Fig. 2). The nuclei were arranged either around the periphery of the cell or were scattered irregularly throughout the cytoplasm. They were small rounded nuclei, staining darkly with haematoxylin and resembling those of the spermatids. A few contained single small vacuoles similar to those present at this stage in the nuclei of spermatids ; however, in some of the latter 2 or even 3 distinctly separate intranuclear vacuoles were seen whereas none of the giant cell nuclei showed more than one vacuole. Such giant cells appeared to correspond to the spermatid stage of development. A number were seen in various degrees of degeneration with clumping and fragmentation of nuclear material together with disintegration of the cytoplasm. Degenerated spermatids with vacuolated nuclei were present in large numbers and appear to be similar to those reported by Alvizouri and Warren (1954) following ethionine administration. Within the tubules large vacuoles now appeared between the cell, probably due to absorption of necrotic cellular material (Fig. 2).

By the end of 48 hr. there was extensive damage within the tubules, and by 96 hr. destruction of cells within the tubules was complete (Fig. 3). At this dosage level the Sertoli cells were involved. The tubules were still of normal size and outline but now contained masses of amorphous eosinophilic material with occasional deposits of fragmented nuclear material. Spermatozoa were still clearly recognisable morphologically.

Leydig and other cells of the interstitial tissue were unaffected and survived in both the centre and periphery of the testis, except in some instances where a higher dose of cadmium was given and in these latter there was complete destruction of all testicular tissue within the tunica albuginea. In this series however,

the interstitial cells survived and by the twenty-first day there was histological evidence of cellular proliferation in scattered islands (Fig. 3).

A macroscopic examination at autopsy of other organs revealed no gross changes. However, small lesions were found in histological sections of liver from 3 cases. In one (30 hr. after injection) this amounted only to a moderate degree of fatty change in some lobules, mostly confined to the middle and central zones. In the other two (96 hr.) necrotic lesions were irregularly distributed throughout the liver. Their outlines were fairly sharply defined and they were situated in the midzonal region (Fig. 4). Within these areas there was necrosis of all cells. No distinctive nuclear changes could be determined owing to the advanced state of these lesions. Histological examination of bone marrow, pancreas and prostate (taken from a small number of animals only) showed no recognisable changes.

DISCUSSION

It is evident from these results that cadmium is highly toxic to cells of the seminiferous epithelium.

With high doses the entire mass of tissue within the tunica albuginea was destroyed ; with smaller doses there was survival of intertubular islands of Leydig cells, fibroblasts and associated blood vessels yet complete destruction of neighbouring tubular epithelium. This suggests either that the spermatogenic cells are much more sensitive to the action of cadmium than the other cells, or that the involvement of the interstitial cells is secondary to the sudden release of large quantities of cellular breakdown products in the immediate vicinity. The latter is considered to be the more likely explanation. No damage to the epithelium of epididymis has been seen.

It will be recalled that White (1955) investigated the action of a number of heavy metals on the motility of ram, bull, rabbit and human spermatozoa. He found considerable species differences in response, and reported cadmium to be highly toxic in this respect to human spermatozoa, and to a much less degree to those of bull.

It is possible that cadmium exerts this action most effectively on cells in or about to enter mitosis, and if so one would not expect any obvious lesions in liver where the mitotic rate is normally low. However, liver cell damage occurred in a small number of these animals, and this finding is in agreement with that of Parizek (1957) who noted it occasionally. This observation, taken with the absence of recognisable lesions in bone marrow, suggests that cadmium damage is not necessarily confined to tissues with a high mitotic rate, and neither need it affect all such tissues. There is therefore no reason to suppose a special relationship to some particular stage of mitosis from this evidence.

In view of the selective toxicity shown by cadmium to cells of the seminiferous epithelium it would be of interest to know whether a similar effect is exerted on primary neoplasms arising from this type of cell.

SUMMARY

Young adult mice received a single dose of cadmium chloride which produced extensive testicular damage with total destruction of all tubular cells within 96 hr. and occasionally some midzonal lesions in the liver.

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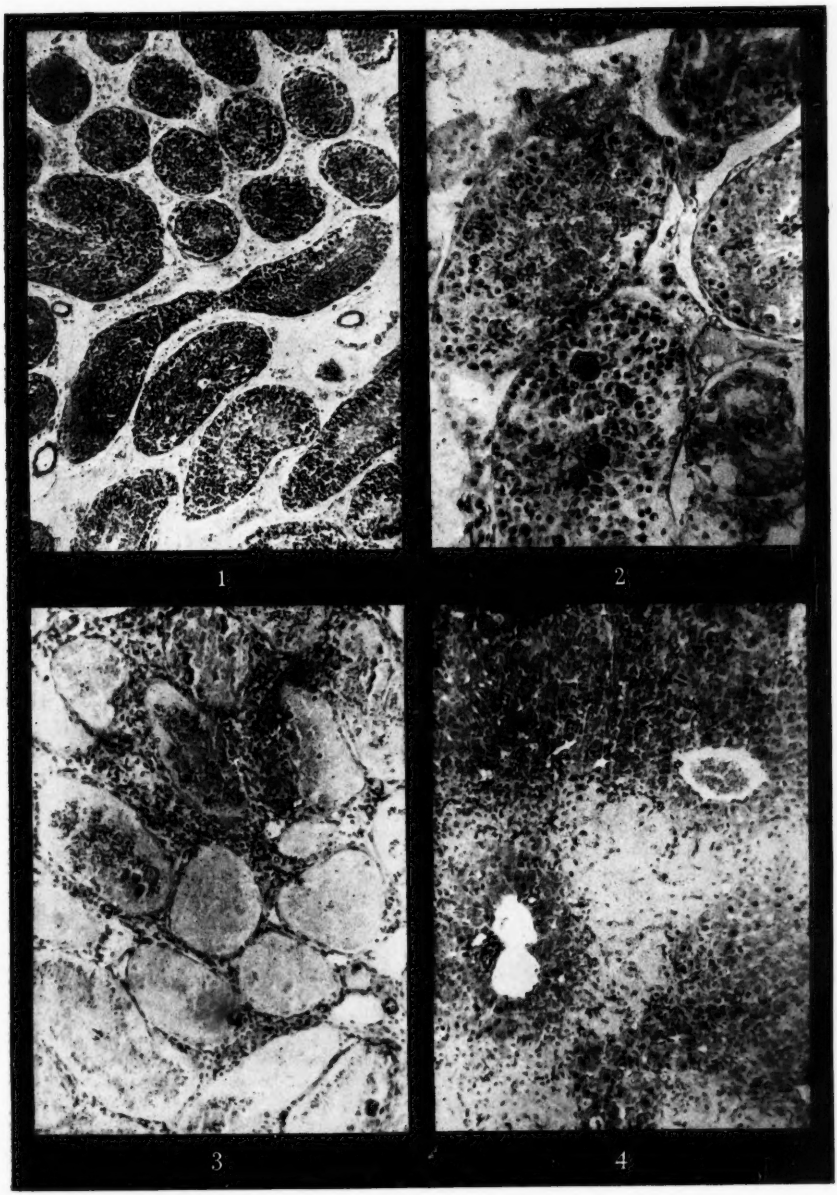
EXPLANATION OF PLATE

FIG. 1.—Testis at 16 hr. showing gross interstitial oedema. San Felice. Haematoxylin and Eosin.
FIG. 2.—Testis at 48 hr. showing giant cells. San Felice. Haematoxylin and Eosin.
FIG. 3.—Testis at 21 days with complete destruction of tubular cells and early proliferation of interstitial tissue. San Felice. Haematoxylin and Eosin.
FIG. 4.—Liver at 96 hr. showing midzonal necrosis. Formalin. Haematoxylin and Eosin.



Meek.

THE BACTERICIDAL ACTIVITY OF MOUSE MACROPHAGES *IN VITRO*

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IT has been shown that many strains of bacteria are rapidly killed in the peritoneum following phagocytosis by mouse macrophages, Whitby and Rowley (1959). It is likely that under these circumstances the effect is nearest to that occurring under natural conditions of infection, but in order to remove some of the ambiguities from these previous findings, the reactions between macrophages and bacteria have now been followed entirely under *in vitro* conditions. The results to be presented here will show that by these *in vitro* methods the bactericidal effects can still be demonstrated, although with the increased manipulations involved, the macrophages are not so active in this respect as they are *in vivo*.

MATERIALS AND METHODS

Strains of bacteria.—The following were used: *Bacterium coli* E2206, E2380 and 145 whose characteristics were described by Rowley (1954), *Salmonella typhimurium* C5 and M206—an avirulent strain (Furness and Rowley, 1956), *Bacterium friedlanderi* strain 2F which was mouse virulent, a recently isolated strain of *Pseudomonas pyocyanea* and the Oxford strain of *Staphylococcus aureus*.

All strains were kept on agar slopes in screw capped bottles at 4° from which they were subcultured as required.

Mouse strain.—All mice used were of the L.A.B. strain of grey mice bred in this Institute. Mice were kept on the standard pellet diet 41B and weighed 18–25 g. when used.

Bacterial antisera.—Bacterial antisera were prepared by a standard method (Wadsworth, 1927). When used for opsonizing bacteria they were diluted to a strength 5 times greater than the final agglutinating titre. Agglutination tests on sera were performed by the method of Felix and Bensted (1954). All sera were heated at 56° for 30 min. before use.

Tissue Culture Methods

Flasks.—Porter flasks (Porter, Claude and Fullam, 1945) made of neutral glass and of 3·5 cm. internal diameter were used for all cultures, into each of these a "flying coverslip" was inserted. All glassware was cleaned in hot Calgon and sodium metasilicate solution followed by prolonged rinsing in distilled water as described by Hanks (1955).

Medium.—Medium No. 199 (Morgan, Morton and Parker, 1950) kindly supplied by the Biological Standards department of the M.R.C. was used. This medium contained no antibiotic and was adjusted to pH 7·2 by adding fresh 1 per cent sodium bicarbonate solution. On receipt the medium was dispensed into screw capped 20 ml. pyrex glass tubes and stored at 4°.

Peritoneal macrophages.—In the previous paper (Whitby and Rowley, 1959) macrophages were infected with organisms whilst still in the mouse peritoneum and were subsequently transferred to tissue culture flasks. While this involved the minimum handling of the peri-

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toneal cells the method had the disadvantage that phagocytosis and bacterial killing occurred in the peritoneum and continued when the cells were transferred to the *in vitro* system. This complicated the calculation of the bactericidal rates due to the uncertainty of the time at which phagocytosis had occurred. For this reason we have endeavoured to simplify the situation by two distinct steps. In the first method (Type I) macrophages have been infected *in vitro* and the numbers of bacteria surviving inside and outside the cells have been determined at intervals without further treatment. In the second method (Type II expts.) the macrophage cultures have also been infected *in vitro* and after a certain period the unphagocytosed bacteria remaining in the extracellular fluid have been washed away, leaving behind a culture of infected macrophages bathed in fresh medium on which intracellular bacterial counts could be performed at intervals. This type II method has the theoretical advantage that the intracellular bactericidal rate can be studied free from the interference of continued phagocytosis and extracellular bacterial multiplication.

For both these types of experiment the mice were killed and the peritoneum washed out aseptically with 1·5 ml. of 199 medium + 0·5 i.u./ml. Heparin as described previously (Whitby and Rowley, 1959). 1 ml. of this peritoneal eluate which contained approximately 4×10^6 macrophages was transferred to a Porter flask containing a " flying coverslip ", the flask was then flushed out with a 5 per cent CO_2/air mixture, stoppered and incubated at 37°.

Type I Experiment.—After at least 3 hr. incubation the heparinised medium was sucked out of the flasks and 1 ml. of fresh warmed 199 added, containing the required number of opsonised bacteria. Flasks were taken out of the incubator at intervals, and an aliquot of the supernatant medium plated out on nutrient agar for counting. The " flying coverslip " which occupied $\frac{1}{6}$ of the floor area of the flask was removed aseptically with forceps, inverted on to a plate of MacConkey's medium and spread around vigorously to aid in disruption of the macrophages.

Type II Experiment.—The flasks containing the monolayers of peritoneal cells were infected as in the type I series. After 30 min. the supernatant medium from each flask containing unphagocytosed organisms was removed and counted, (SN) this operation occupied about 5 min. for a series of ten flasks. 1 ml. of fresh 199 medium was now added to all the flasks, which were then incubated and sample flasks were removed at this time and at intervals thereafter. The supernatant and coverslip were then plated and counted as in the type I experiments. In type II experiments each flask acts as a control to the others, for the numbers of unphagocytosed organisms (SN) at the end of the 30 min. infection period should be approximately the same in all flasks (see Table IV). Moreover, the numbers of organisms in the second supernatants should be small by comparison with SN and equal in all cases except where extracellular growth has occurred.

RESULTS

Type I Experiment

The data from a typical experiment are shown in Table I. In this experiment a dilution of the overnight bacterial culture was treated with heated specific rabbit antiserum at five times the limiting agglutinating strength, or with 10 per cent of normal rabbit serum for 30 min. The suspension was further diluted one thousandfold in fresh 199 medium and 1 ml. of this suspension containing an estimated 1–2,000 organisms was pipetted into each flask of macrophages from which the original 199 + heparin had been removed.

The organisms pretreated with immune serum were rapidly removed from the supernatant fluid by the cells so that by 70 min. 77 per cent of the bacteria inoculated had disappeared into the macrophages. Only 31 per cent of the phagocytosed bacteria were viable at this time. These considerations certainly underestimate the bactericidal effect since control flasks inoculated with 199 medium without macrophages revealed that considerable bacterial growth had occurred during this time. It is probable therefore that the numbers of bacteria phagocy-

tosed were greater than that implied by the calculations based on the initial inoculum. By contrast to the above results the bacteria pretreated with normal rabbit serum were phagocytosed to such a small extent that the bacterial counts in the supernatant fluid increased steadily. In Fig. I the total recovery of organisms in each flask is plotted as a percentage of the inoculum. The bactericidal activities of the macrophages were such that 70 min. after inoculation only 47 per cent of the inoculum could be recovered from the flask, after this time the rapid extracellular growth of the *Bact. coli* organisms swamped the phagocytic and bactericidal properties of the system.

A rate of killing or " half life " of the phagocytosed organisms has been calculated on the assumptions that the total numbers of organisms phagocytosed

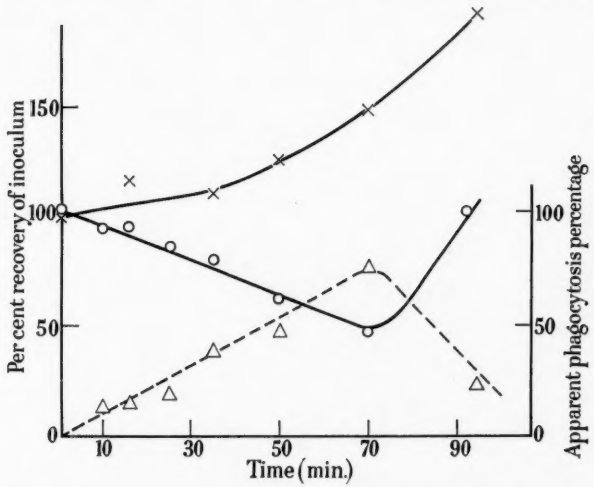

FIG. 1.—Type I expt. Recovery of antiserum treated *Bact. coli* E2380 from macrophages. ○—○ Flask containing organisms and macrophages. ×—× Flask containing organisms only. △ - - △ Apparent phagocytosis (assuming no bacterial multiplication).

were intracellular at the start of the experiment and that the bactericidal effect had lasted from the start until the time of sampling—this is obviously only an approximation, but gives an indication of the rate of killing.

Similar experiments were made with a variety of organisms and the data collected in Table II show that the macrophages were in all cases able to kill the bacteria. The rates of these bactericidal effects, as revealed by the times at which 50 per cent of the intracellular bacteria have been killed (half life) are of a similar order from one strain to another. The data on two experiments with *Bact. coli* 145 show as great differences as do those between different organisms. In particular there is no significant difference between the rates of killing of the two strains of *Salm. typhimurium* one of which (C_5) is highly virulent, whereas the other (M_{206}) is not.

Macrophages from Specifically Immune Mice.—A group of 50 mice were given three injections of heat killed vaccine of *Bact. coli* E2380 at intervals of 3 days and used as the source of peritoneal macrophages 12 days after the last injection. The

TABLE I.—*The Recovery of* Bact. coli E2380 *at Intervals after Inoculation into Flasks Containing Peritoneal Macrophages*

Time (min.)	SN Orgs. in supernatant	P Phagocytosed (by diff.)	C Orgs. in cells	SN + C total	Total recovery (per cent)	$\frac{C}{P} + 100$ per cent viable	Half life of intracellular orgs. (min.)	Control flasks without macrophages: SN	Control flasks without macrophages: Recovery (per cent)
A. Inoculum into each flask 1200 organisms, opsonised with immune serum									
0	1190	10	40	1230	103	400	—	1200	100
10	1050	150	75	1125	94	50	10	—	—
16	1020	180	105	1125	94	58	21	1390	116
25	970	230	55	1025	86	24	12·5	—	—
35	750	450	205	955	80	45	29	1320	110
50	630	570	115	745	62	20	21	1500	125
70	280	920	290	570	47	31	31	1750	146
95	910	290	325	1235	103	112	—	2300	192
B. Inoculum into each flask 1500 organisms, pretreated with normal rabbit serum									
2	1420	80	45	1465	97	—	—	1500	100
8	1650	—	35	1685	112	—	—	—	—
15	1720	—	135	1755	117	—	—	—	—
20	1550	—	90	1640	109	—	—	1650	110
30	1540	—	130	1670	111	—	—	1800	120
45	2120	—	500	2620	174	—	—	2110	141
65	2430	—	265	2695	179	—	—	2880	190
90	3460	—	470	3930	262	—	—	3300	220

TABLE II.—*Accumulated Data on Bactericidal Activity of Mouse Macrophages with Different Bactericidal Strains. Type I Experiments*

Organism	Bacteria inoculated per flask	Time for 50 per cent phagocytosis (min.)	Half life once 20 per cent phagocytosis had been achieved (min.): Mean	Half life once 20 per cent phagocytosis had been achieved (min.): Range
Ps. pyocyanea	5100	30	23	(13–36)
	4900	—	12	(3–25)
Bact. coli E2206	5800	65	13	(5–23)
	2000	60	14	(5–26)
Bact. coli E2380	1200	55	20	(10–31)
Bact. coli 145	4250	50	35	(12–53)
	2500	45	15	(10–21)
Salm. typhimurium M206	4200	25	22	(10–29)
Salm. typhimurium C5	2500	40	18	(4–41)
Bact. friedlander	3500	30	37	(10–70)
Staph. aureus	900	40	—	—
Non-specifically Immune Mice				
Salm. typhimurium C5	2300	30	9	(2–18)
Bact. coli E2380	2650	40	19	(5–52)
Specifically Immune Mice				
Bact. coli E2380	2650	50	17	(5–35)

pooled serum from 10 of these animals gave an agglutin titre of 1/100 against the homologous organism. The macrophage preparations were infected with *Bact. coli* E2380, previously opsonized with antiserum, as in the experiments above. There was no clear evidence from this experiment that the macrophages from specifically immune mice were any more effective in destroying fully-opsonized bacteria than were normal macrophages (Table III and Fig. 2).

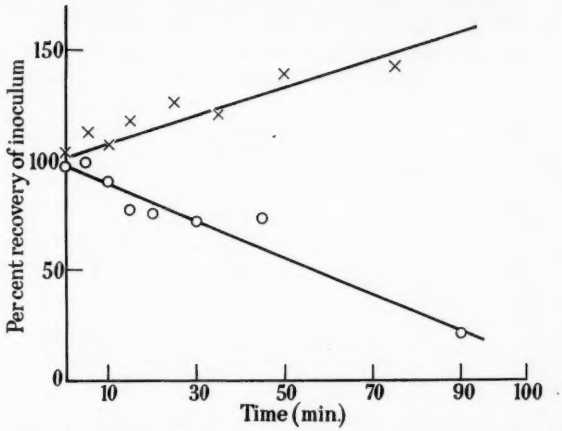

FIG. 2.—Type I expt. Recovery of *Bact. coli* E2380 from macrophages from immune mice. ×——× Organisms treated with normal serum. ○——○ Organisms treated with immune serum.

TABLE III.—*Recovery of* Bact. coli *E2380 at Intervals after Inoculation into Flasks Containing Peritoneal Macrophages from Specifically Immune Mice. Inoculum 2650 Organisms into Each Flask.*

Time (min.)	SN Orgs. in supernatant	P Phagocytosed (by difference)	C Orgs. in cells	Total recovery (per cent)	$\frac{C}{P} \times 100$ per cent viable	Half life of intracellular orgs. (min.)
0	2500	150	75	97	50	—
5	2500	150	125	99	83	—
10	2130	520	150	86	29	5
15	1790	860	165	74	19	6
20	1890	760	135	77	18	8
30	1470	1180	425	71	36	20
45	1410	1240	560	73	45	35
90	230	2420	315	21	13	30

Macrophages from mice given lipopolysaccharide.—Fifty mice were injected intravenously with 0·2 ml. of a solution containing 50 μg. lipopolysaccharide prepared from *Bact. coli* E2206. After 48 hr. they were used to provide macrophages for Type I experiments against *Salm. typhimurium* C_5 and against *Bact. coli* E2380. The pooled serum from a representative group of these animals did not possess any agglutinating antibodies against either of these strains. The bactericidal activities of these macrophages against the fully opsonized bacteria did not significantly differ from the rates with normal cells (Table II).

Type II Experiments

A typical experiment is tabulated in Table IV. Each of the ten flasks of macrophages were inoculated with 1,200 *Salm. typhimurium* M206 which had been opsonized with antiserum. Thirty minutes later the supernatant fluid was removed, 0·1 ml. plated for viable count and 1 ml. of fresh 199 medium added to each flask. Two flasks were used immediately to count the viable organisms in the cells, and the other flasks were similarly treated after varying times of incubation. The detailed results are shown in Table IV, from which it can be seen that in the individual flasks the macrophages behaved in a surprisingly uniform fashion so far as the amount of phagocytosis during the initial period was concerned.

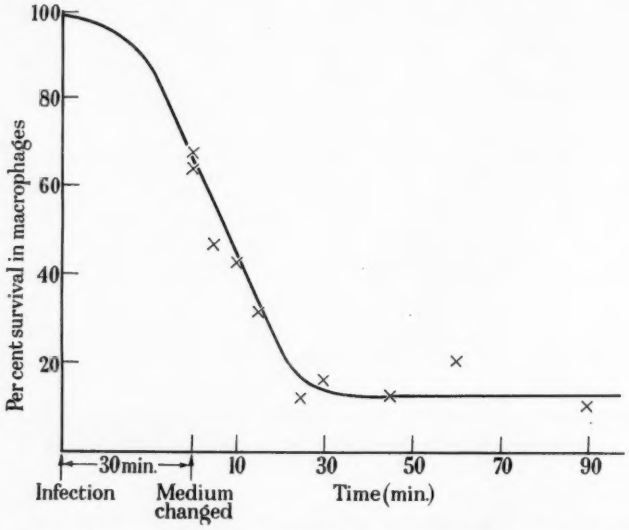

FIG. 3.—Type II expt. Recovery of viable *Salm. typhimurium* from macrophages at various times after phagocytosis.

The numbers of organisms recovered from the macrophages decreased with time of incubation. The times in the table commence with the addition of the fresh medium to the flasks, so that the finding that only 66 per cent of the intracellular organisms were surviving at zero time can be explained by bactericidal activity occurring during the 30 min. of initial contact. To calculate the rate of intracellular bacterial killing one can assume that at zero time all the flasks would have the same percentage of surviving intracellular bacteria as in the flask sampled at zero time *i.e.* 66 per cent and follow the subsequent decrease in viable count from this level. Alternatively, one can assume that the rate of phagocytosis was never a limiting factor and calculate the " half life " from the moment of infection. The results are of a similar order irrespective of the method of calculation and agree quite reasonably with the rates found in the Type I experiments. In Fig. 3 the survival of the intracellular organisms is plotted against time of incubation. Similar results were obtained with the other strains of micro-organisms

tested and the data are summarized in Table V where the "half lives" of the intracellular bacteria have been calculated by both methods, where possible.

TABLE IV.—*Recovery of* Salm. typhi-murium *M206 from Mouse Macrophages where after ½ hr. the Supernatant Fluid (Containing Unphagocytosed Bacteria) was Removed and Replaced by Fresh Medium (Type II Experiment). Inoculum into each Flask* 1,200 *Organisms, Opsonized with Anti-Serum*

T After removal of SN time	SN Supernatant at end of period of phagocytosis	P Phagocytosis by difference	C Organisms recovered from cells	$\frac{C}{P} \times 100$ per cent survival in phagocytes	Half life of phagocytosed organisms min. (Time = 30 min. + T)	Half life assuming that after removal of supernatant 66 per cent of phagocytosed bacteria are alive
0	460	740	505	68	50	—
0	490	710	455	64	44	—
5	370	830	390	47	31	10
10	450	750	325	43	31	16
15	520	680	220	32	27	15
25	440	760	90	12	18	10
30	330	870	140	16	22	15
45	270	930	120	13	25	19
60	450	750	160	21	40	36
90	300	900	100	11	37	36

Mean phagocytosis = 792
Range 930–680

TABLE V.—*Accumulated Data on Bactericidal Activity of Mouse Macrophages with Different Bactericidal Strains. Type II Experiments*

Organism	Phagocytosis at 30 min. mean (per cent)	Mean number of organisms phagocytosed	Half life from time of infection (min.) Mean	Half life from time of infection (min.) Range	Half life from time of removal of SN (min.) Mean	Half life from time of removal of SN (min.) Range
Ps. pyocyanea	63	2500	21	(14–30)	14	(9–22)
	18	1000	32	(19–56)	25	(13–55)
Bact. coli E2206	29	480	29	(17–36)	16	(11–22)
	25(10 min.)	560	23	(13–48)	22	(8–62)
Bact. coli E2380	60	12000	11	(7–21)	17	(8–30)
	41	1100	23	(18–29)	35	(22–54)
Bact. coli 145	29	720	29	(15–40)	15	(7–20)
Salm. typhimurium M206	66	790	33	(18–50)	20	(10–36)
Salm. typhimurium CS	46	2850	11	(8–21)	17	(4–42)
Bact. friedlanderi	53	3450	32	(14–57)	12	(4–34)
Staph. aureus	80	1200	61	(41–106)		—
	48	2400	39	(26–51)	17	(8–30)
Bact. coli 145	20	460	46	(27–66)		—

DISCUSSION

Although the experimental methods used here of infecting the macrophages *in vitro* leave fewer ambiguities in the interpretation of the results than did the method involving *in vivo* infection, nevertheless the methods have disadvantages. The chief among these is the relative inefficiency of phagocytosis by the macrophage monolayers of bacteria which are in a supernatant layer of fluid, probably 1,000 times the depth of the macrophages. This necessitates a longer time of contact

for sufficient phagocytosis to occur and since bacterial growth is taking place concurrently there are many opportunities for differences to develop from flask to flask. In spite of this the overall conclusion is quite clear. The macrophages are rapidly bactericidal for all the strains of organisms examined and there are no obvious correlations between the rates of killing and the virulence of the organisms. Where larger numbers of bacteria are used to infect the macrophage cultures, the method of colony counting adopted may become less efficient owing to coincidence of bacterial colonies. This probably explains why the rate of killing of *Bact. coli* E2380 detailed in Table V was found to be twice as fast where 12,000 organisms were phagocytosed per flask as compared with the rate given with 1,100 organisms per flask.

The question arises when such large numbers of peritoneal cells are infected with small numbers of bacteria whether the results obtained might not be due to the small percentage of polymorphonuclear leucocytes in the peritoneal eluates. To overcome this difficulty we have conducted several experiments with peritoneal cells which have been kept in flasks for 24 hr. or longer before infection. Under these conditions the polymorphs fall off the glass and the population is essentially a pure culture of macrophages. The results with such cell layers were very similar to those reported earlier and confirm that it is the bactericidal activity of macrophages in the cell population which we have been measuring.

In this work we have avoided the use of antibiotics so that such bactericidal activities as we have here observed cannot be due to antibiotics. On the other hand this makes the comparison between the present work and that of other authors difficult, since our results can only extend over the first two hours of the bacterium macrophage interaction due to bacterial growth in the extracellular medium. In a recent paper by Furness (1958) mouse macrophages in the presence of neomycin were found to be equally bactericidal during the first 3 hr. of contact for the same pair of virulent and avirulent strains of *Salm. typhimurium* used here. Subsequently the virulent organisms multiplied rapidly and killed the macrophages, and Furness concluded that the virulence of a strain of *Salm. typhimurium* depended on its ability to multiply in macrophages and kill them, which avirulent strains were unable to do. It is not possible to deduce the rate of the bactericidal process from Furness' paper, since the first reading was taken at 3 hr. Under the conditions of our experiments it seems clear that once the organisms have been phagocytosed there is not much difference in the rate of killing of virulent or avirulent strains by mouse macrophages.

In an earlier paper Rowley (1958) described the technique used here and showed that bacteria which had not been in contact with serum were able to grow inside macrophages whereas serum treated bacteria were rapidly killed. The present results throw no further light on this interesting finding since all the work reported here has been in the presence of serum.

SUMMARY

A simple method is described whereby macrophages from the mouse peritoneum may be infected with bacteria *in vitro*, and the fate of the bacteria followed after phagocytosis. Using this system it has been demonstrated that opsonized bacteria were phagocytosed and killed by the macrophages. Unopsonized bacteria were not phagocytosed.

There was no correlation between the rate of killing of different organisms and their virulence for mice, all gram negative organisms tested being killed at a similar rate.

Macrophages from specifically immune animals and from those with an enhanced degree of non-specific immunity behaved like normal macrophages.

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SURVEY OF PAPERS

GINSBURG has investigated the action of streptococcal haemolysins on Ehrlich ascites tumour cells, alone and in combination with various proteolytic enzymes. He found that the haemolysins apparently killed the cells. Cells not attacked by such proteolytic enzymes as trypsin, were disintegrated by the enzymes after being exposed to streptococcal haemolysins. In the case of streptolysin O, this effect was abolished by ascitic fluid and rabbit serum (p. 417).

MACKANESS has measured the concentration of hypertensinogen in normal and pregnant rats. He found that the blood level fell significantly in late pregnancy. Reasons are given for the belief that the diminished response of pregnant rats to renin may be due to the low blood hypertensinogen levels (p. 424).

OLITZKI, using the Ouchterlony technique, tested antisera prepared in rabbits by the injection of acetone-killed Brucella organisms, against antigens prepared by disintegrating bacteria of that genus. Quantitative titrations and absorption tests showed that *Br. abortus*, *Br. melitensis* and *Br. Suis* possess at least 6 soluble antigens which differ in concentration in the species, but no antigen was specific for a single species (p. 432).

BRUMFITT has analysed cell walls prepared by sonic disruption of *Micrococcus Lysodeikticus* of strains sensitive and resistant to lysozyme. He found O-acetyl groups accompanied resistance, and their removal led to sensitivity, but this difference did not account for the natural resistance to lysozyme of *Staphylococcus aureus* (p. 441).

LE BOUVIER examined by precipitation in agar the antigens associated with sucrose-density-gradient fractions C and D of purified poliovirus suspensions. Antisera to tissue culture grown virus suspensions contained precipitins to C and D, the precipitation lines with these antigens being continuous with the minor and major lines respectively, obtained with unfractionated homotypic poliovirus suspensions. Heating antigen D rendered it serologically indistinguishable from C. Heating unfractionated suspensions destroyed D and increased the amount of C present. Unheated virus suspensions injected into rabbits produced neutralizing antibodies, anti-D and anti-C precipitins; heated virus led to the formation of anti-C precipitin only (p. 452).

JULIA FOURMAN investigated the sites of nephrocalcinosis in rats produced by intraperitoneal injections of calcium gluconate or sodium phosphate. The daily peritoneal injection of calcium gluconate raised the serum, urine and renal calcium levels, and calcium was deposited on the basement membranes of the proximal convoluted tubules and nowhere else.

Repeated intraperitoneal injection of sodium phosphate, by contrast, did not raise the serum phosphate but increased the excretion of calcium and phosphate in the urine. Calcium was deposited in the kidneys in a variable manner involving the basement membranes of the second part of the proximal convoluted tubules, Henle's loop, the small collecting ducts and later in the glomerular tufts. The author regards the nephrocalcium produced by calcium gluconate as due to the raised calcium content of the urine and serum, while the nephrocalcium following phosphate injections as due to secondary changes following renal damage induced by increased phosphate excretion (p. 464).

JENKIN and ROWLEY have tested the effect of various toxic fractions of *Vibrio cholerae* by injecting them into ligated loops of rabbit small intestine. They found factors concerned in the pathogenesis of cholera, the most important of which was lactic acid produced by the organism during growth (p. 474).

AUSTEN, BECKER and MARCUS, using the esters TAMe and LMe as substrates, examined plasmin esterases in guinea-pig serum activated by human euglobulin and streptokinase only, or by these substances preceded by chloroform or peptone. They concluded that they had activated 2 plasminogens ; C, found in chloroform-treated serum, heat-stable and producing an esterase inhibited by soybean, and active on TAMe and LMe ; and P, found in peptone treated serum, heat labile, with a product resistant to soybean and active on TAMe. Neither was affected by an antigen-antibody precipitate (p. 482).

BECKER, AUSTEN and MARCUS, continuing to examine serum esterases, describe two which are activated by peptone and resistant to soybean inhibitor. One arising from a heat labile precursor is more active against TAMe than ATEe, works best at pH 8·0 and is absorbable by antigen-antibody complexes ; the other is not thus absorbable, is more active at pH 9·0 and against ATEe and derives from a heat stable proenzyme. The former resembles, but cannot certainly be identified with, the first component of complement (p. 494).

MEEK has inoculated male mice with a single dose of cadmium chloride and examined the effects on the testes and liver histologically after various intervals. He found that cadmium was highly toxic to cells of the seminiferous epithelium, high doses destroying the entire mass of tissue within the tunica albuginea ; with smaller doses there was survival of intertubular islands of Leydig cells, fibroblasts and blood vessels. Occasionally he found midzonal liver lesions (p. 503).

ROWLEY and WHITBY have developed a method of assessing the bactericidal activity of mouse macrophages by a complete *in vitro* technique. They found that opsonised bacteria were phagocytosed and killed by macrophages, while unopsonised bacteria were not affected. There was no correlation between the rate of killing of different organisms by the macrophages and their virulence for mice (p. 507).

ADDENDUM

Vol. XL, No. 5, October 1959 (W. Brumfitt), P. 447, Fig. 7.
Key: 1. Glutamic acid. 2. Glycine. 3. Alanine. 4. Lysine.
5. Glucosamine. 6. Muramic acid.